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AWARD NUMBER DAMD17-96-1-6085

TITLE: Identification of Components of the Cell Death Pathway

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REPORT DATE: June 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

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1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 1998	Annual (1 Jun 97 - 31 May 98)

4. TITLE AND SUBTITLE
Identification of Components of the Cell Death Pathway

5. FUNDING NUMBERS
DAMD17-96-1-6085

6. AUTHOR(S)
Vishva M. Dixit, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Michigan Hospitals
Ann Arbor, Michigan 48109-0602

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

19990407 085

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Transformed cells retain part or all their apoptotic machinery. Therefore, understanding the molecular details of this machinery is paramount for developing new anti cancer therapies that activate the cells own suicide program. The lab has focused initially on the identification of cell death components that are activated by the cell surface receptors FAS and TNFR-1. This has led to identification of the Caspase cascade. Remarkably, the some members of the cascade are directly recruited to the receptors. The activation of the apical Caspase is the first enzymatic reaction during death receptor signaling and we show that this reaction is auto-catalytic triggered by aggregation of the zymogen form of Caspase-8. We identified a protein homologous to Caspase-8 that inhibits auto-activation. It has no proteolytic activity and binds to Caspase-8 inhibiting receptor recruitment and hence auto-activation. Caspase-9 is another apical Caspase that is implicated to be activated by intracellular death stimuli and associates with Apaf-1. We show that molecular interactions observed in *C. elegans* are conserved in mammalian cells. Caspase-9 forms a complex with Apaf-1 and Bcl-x_L. This observation uncovers a potential mechanism for the Bcl-2 family of proteins. In addition, we have identified two new Caspases. The large number of Caspases highlights the complexity of the system but also allows for specific therapeutic intervention.

14. SUBJECT TERMS
Breast Cancer, Apoptosis, Caspase, Bcl-X2

15. NUMBER OF PAGES 65

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction:

The critical role of apoptosis during development, tissue homeostasis, and pathological conditions is undisputed. Significant advances have been made during the last 5 years in the elucidation of the molecular mechanisms involved. Clearly, apoptotic mechanisms are conserved between species and parts of the same pathway are utilized for a multitude of stimuli that induce apoptosis. Most significant for cancer research is the realization that this common apoptotic machinery is still intact in transformed cells, including breast cancer cells. Therefore, the development of pharmacological means to specifically trigger the process has promise to become a new anti cancer strategy.

The objective of the grant application was to delineate in biochemical terms components of the death pathway, especially cysteine proteases belonging to the caspase family that form the effector arm of the death pathway.

We initially focused our studies on the activation of these caspases by signals emanating from the death receptors Fas (CD-95/Apo1) and TNFR-1. These studies led to the identification of death receptor associated caspases (FLICE, FLICE2) and established the existence of a caspase cascade. A significant amount of these results were produced in this lab and have been summarized in last years progress report.

As a consequence of this work the number of identified caspases has quickly grown. We have made use of the homology information to identify two additional caspases, ERICE (Caspase-13) and MICE (Caspase-14).

We also identified an inhibitor of apoptosis with caspase homology, I-FLICE. This molecule has no active site cysteine but has otherwise significant homology to FLICE (Caspase-8). Most significantly the death effector motif in the pro-domain is conserved as a functional interaction domain.

We have started addressing the question on how the apical caspases are regulated. This was first done with FLICE were we showed that FLICE can be auto-activated by the proximity resulting from receptor recruitment. Caspase-9, another apical caspase cloned in the lab, has been shown to be associated with the human homologue of CED-4, Apaf-1. We show that, in analogy to the molecular interactions observed in *C. elegans*, Apaf-1 mediates the interaction of Bcl-x_L with Caspase-9. This suggests a mechanism by which Caspase-9 activity is directly regulated by anti-apoptotic Bcl-2 family members.

The mechanistic picture that emerges is that caspases represent the common part of the pathway used by almost all apoptotic stimuli. The next question is how do such disparate apoptotic signals like growth factor withdrawal and ionizing radiation feed into this pathway. An important component of this broader question is how is the activity of the caspases at the head of the cascade controlled. Caspase-8, containing two death effector domains (DED) in its pro-domain, is one of these caspases.

Body:

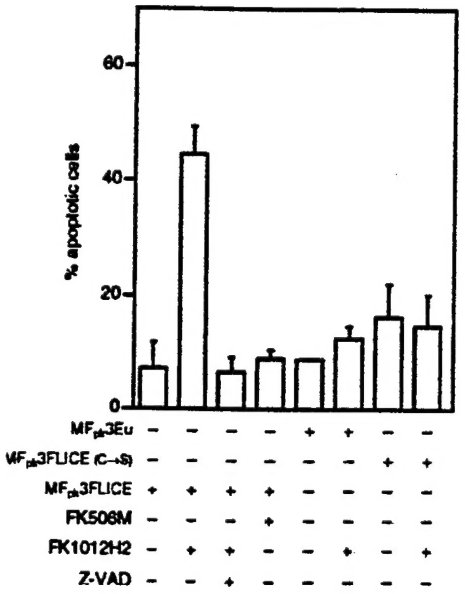
1) Studies on the activation and inhibition mechanism of DED containing caspases.

Death effector domains (DED) are homotypic interactions domains that mediate the binding of caspases to the adapter molecule FADD (1-3). This places the DED containing Caspases-8 and 10 at the apex of the proteolytic cascade and their regulation affects all downstream caspases.

First, we investigated a potential activation mechanism of DED caspases. Recruitment of Caspase-8 upon FAS receptor ligation is expected to result in a high local concentration of the pro-enzyme. Such high local concentrations can be mimicked by using fusion proteins between the molecule of interest and the FK506 binding protein. Oligomerization can then be induced in situ or in vivo by adding the drug FK1012H2, a dimer of FK506 (4).

We replaced the pro-domain of Caspase-8 with a FK506 binding protein (MF_{pk}3FLICE). Fig. 1 shows the results obtained with this system in transient 293 transfections. Clearly the MF_{pk}3FLICE induces apoptosis only in the presence of the dimerization drug.

Fig. 1



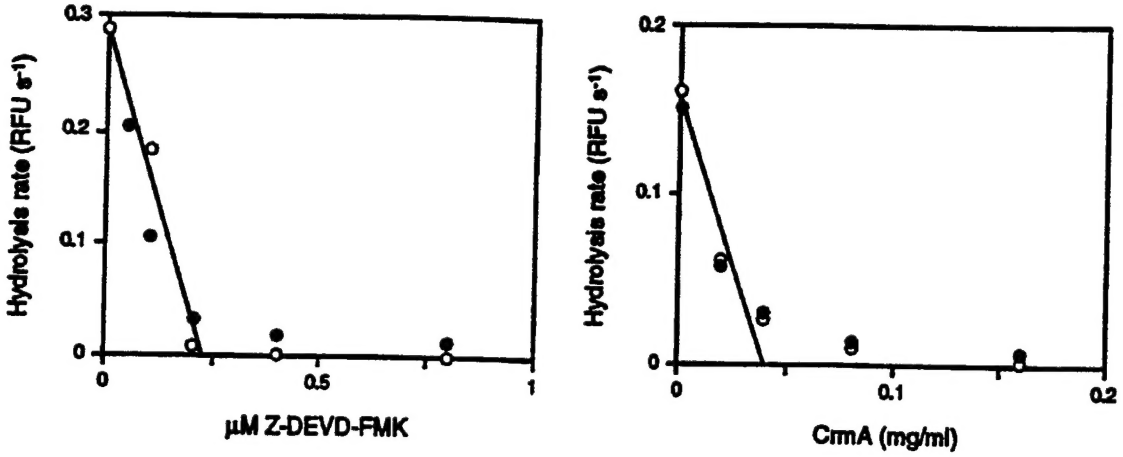
FK1012H2 induces oligomerization of FLICE triggers self-processing and apoptosis.

(D) 293 cells were transiently transfected with $MF_{pk}3EU$, $MF_{pk}3EU3FLICE$, or $MF_{pk}3EU3FLICE$ (C->S) and pCMV-β-Gal. 36 hours post transfection cells were treated with FK1012H2 (250 nM) or FK506M (250 nm) with or without the addition of the broad-spectrum caspase inhibitor z-VAD-fmk (20μM). After 3 hours cells were fixed, stained with X-Gal and examined by phase contrast microscopy. Data (mean+/- s.e.m.) shown are the percentage of round and blebbing apoptotic cells as a function of total number of blue cells counted (n>3).

A prerequisite for proximity alone to trigger zymogen activation is that the unprocessed zymogen has some proteolytic activity. To that end we constructed FLICE cleavage mutants where the aspartic acid residue at the cleavage site has been mutated to an alanine. The resulting protein had a low but significant proteolytic activity with a similar inhibitory profile as the wild type protease, even though it was not proteolytically processed (Fig. 2).

Fig. 2.

b



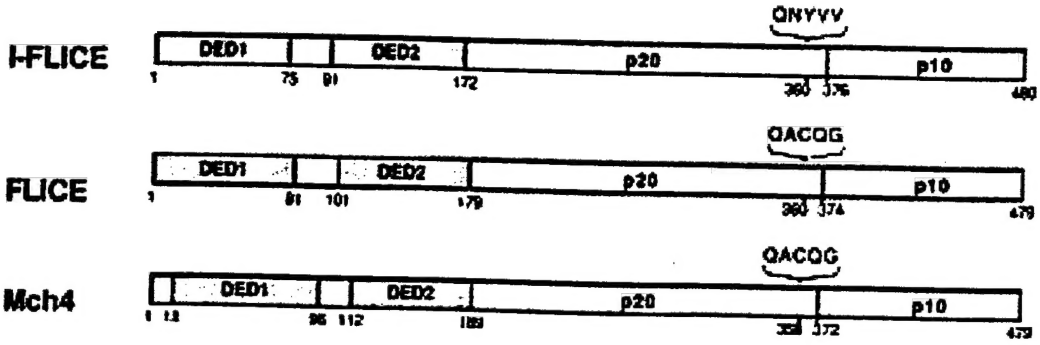
The zymogen form of Caspase-8 has protease activity that is similar to the activated, proteolytically processed, wild type enzyme. *The catalytic domain of Caspase-8 was expressed in pET15b vector. The N-terminal $(His)_6$ tag was used for purification using the QIAexpress Kit (Qiagen). The recombinant protein underwent auto processing during purification into the large and small catalytic domains. A mutant catalytic domain protein was constructed in which the cleavage sites Asp-374 and Asp-384 were modified to Alanines. Purification was achieved as with the wild type protein. As expected the mutant protein was unable to undergo auto-proteolysis during isolation or subsequent incubations. The catalytic activity of each protein was measured by release of AFC (7-amino-4-methyl coumarin) using a Perkin-Elmer LS50 B fluorimeter in a 96 well format. The substrate used was Z-DEVD-AFC. One hundred times more of the mutant enzyme (20 µm) than the wild type protein (0.2 µm) was used to obtain similar activities. Shown are titration experiments with the small molecule inhibitor Z-DEVD-FMK and recombinant CrmA. The relative amount of uninhibited enzyme was evaluated from the initial rates of hydrolysis of the substrate and the concentration of active enzyme was calculated by extrapolating data points to their intersection with the x axis.*

These experiments were mentioned in the last progress report and have now been published in Ref. (5).

This discovery that the zymogen form of Caspase-8 has activity implies that a control mechanism must exist that keeps the enzyme from associating with FADD in the absence of receptor ligation.

We discovered such a molecule that is likely to play a part in this regulation through homology searches with death effector domains. This molecule, termed I-FLICE, has a structure that is strikingly similar to Caspase-8 (Fig. 2).

Fig. 3

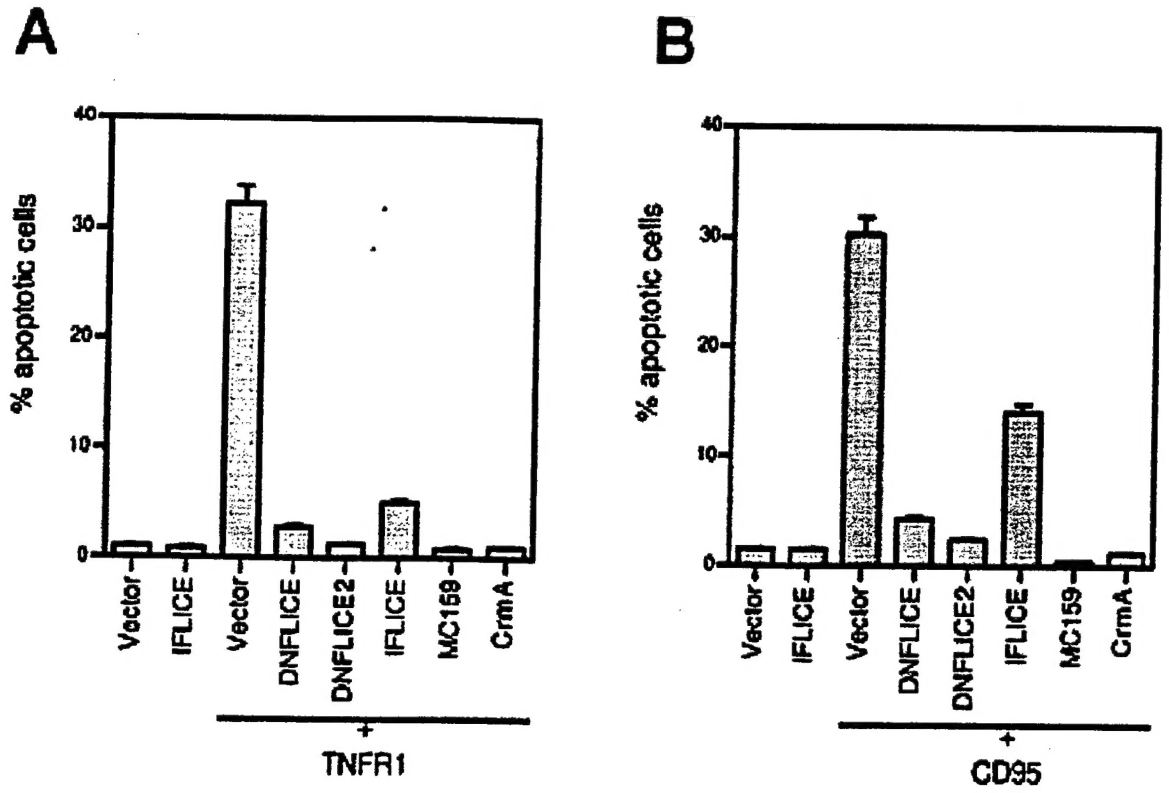


Sequence alignment of I-FLICE with Caspase-8 (FLICE) and Caspase-10 (MCH4).

Schematic models of I-FLICE, FLICE, and Mch4. The N-terminal portion of each molecule contains two DED-like domains (shaded boxes) and the C-terminal portion contains both large and small catalytic subunits (p20/p10). The pentapeptide QNYVV of I-FLICE and the corresponding motifs in FLICE and Mch4 are indicated.

The alignment with Caspase-8 (FLICE) and Caspase-10 (Mch-4) reveals the presence of two death effector domains at the N terminus of the molecule. The death effector domains of I-FLICE were shown to be functional by their ability to bind Caspase-8 and 10. Significant homology between I-FLICE and the catalytic domain of caspases is also revealed in this alignment with some notable exceptions. First, the I-FLICE sequence contains a Tyrosine residue where all caspases have the active site cysteine. Secondly, the residues contacting the Aspartate (P1 position) of the substrate are not present in the I-FLICE sequence. From these observations we predicted that I-FLICE would not have any proteolytic activity and therefore not be able to induce apoptosis. As expected I-FLICE did not induce apoptosis upon overexpression in 293 cells.

Fig. 4



I-FLICE inhibits TNFR-1 and CD95 induced apoptosis
Overexpression of I-FLICE attenuated TNFR-1 (A) and CD-95 (B) induced cell death. 293 (A) or 293-EBNA (B) cells were co-transfected with the indicated plasmids together with the reporter construct pCMV β-galactosidase. The data shown are the percentage of blebbing blue cells as a function of total number of blue cells counted.

I-FLICE overexpression led to significant protection from FAS and TNF receptor induced apoptosis (Fig. 4). We described previously DED containing viral proteins E8 and MC159, collectively called vFLIP, which were potent inhibitors of death receptor induced apoptosis (6). The activity and structure of I-FLICE indicates it to be the cellular homologue of the vFLIP's. The function of I-FLICE is probably more complex because it contains in addition to the DED domains also a partially conserved caspase catalytic domain. In particular the zymogen cleavage site between the large and the small subunit is conserved in I-FLICE suggesting that it can be a substrate for caspases. *In vitro*, I-FLICE is efficiently cleaved by activated Caspase-8. This property is more reminiscent of the viral inhibitors of apoptosis CRMA and p35. Both proteins are cleaved by caspases but the proteolytic products are not released leading to effective inhibition of activated caspases. Only detailed mutational analysis in a physiological context will clarify if the DED domains of I-FLICE act in concert with its partially conserved caspase domain or if different targets are affected by the two domains.

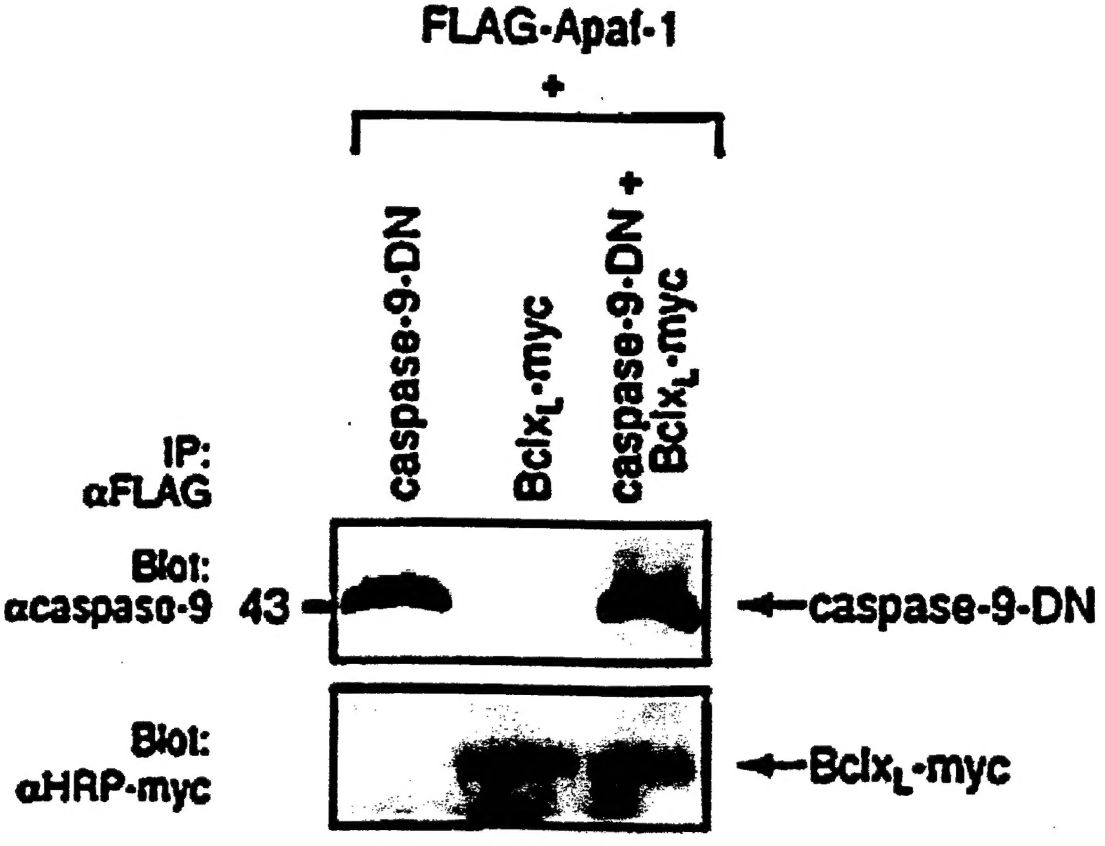
The I-FLICE cloning and characterization was published in Ref. (6)

2) Bcl-X_L function: A role in controlling activation of Caspase-9 by Apaf-1?

We and others had previously shown that the *C. elegans* pro-apoptotic gene products CED-3 and CED-4 form a complex with the anti-apoptotic CED-9 gene (7-9). As the cell death machinery is conserved it is expected that similar interactions play a role in mammalian cells as well. Recently, it was shown that Apaf-1, the mammalian homologue of CED-4, interacts with Caspase-9 and activates this apical caspases in the presence of cytochrome c (10). We were therefore primed to investigate if the mammalian CED-9 homologue, Bcl-X_L, could form similar trimeric complexes.

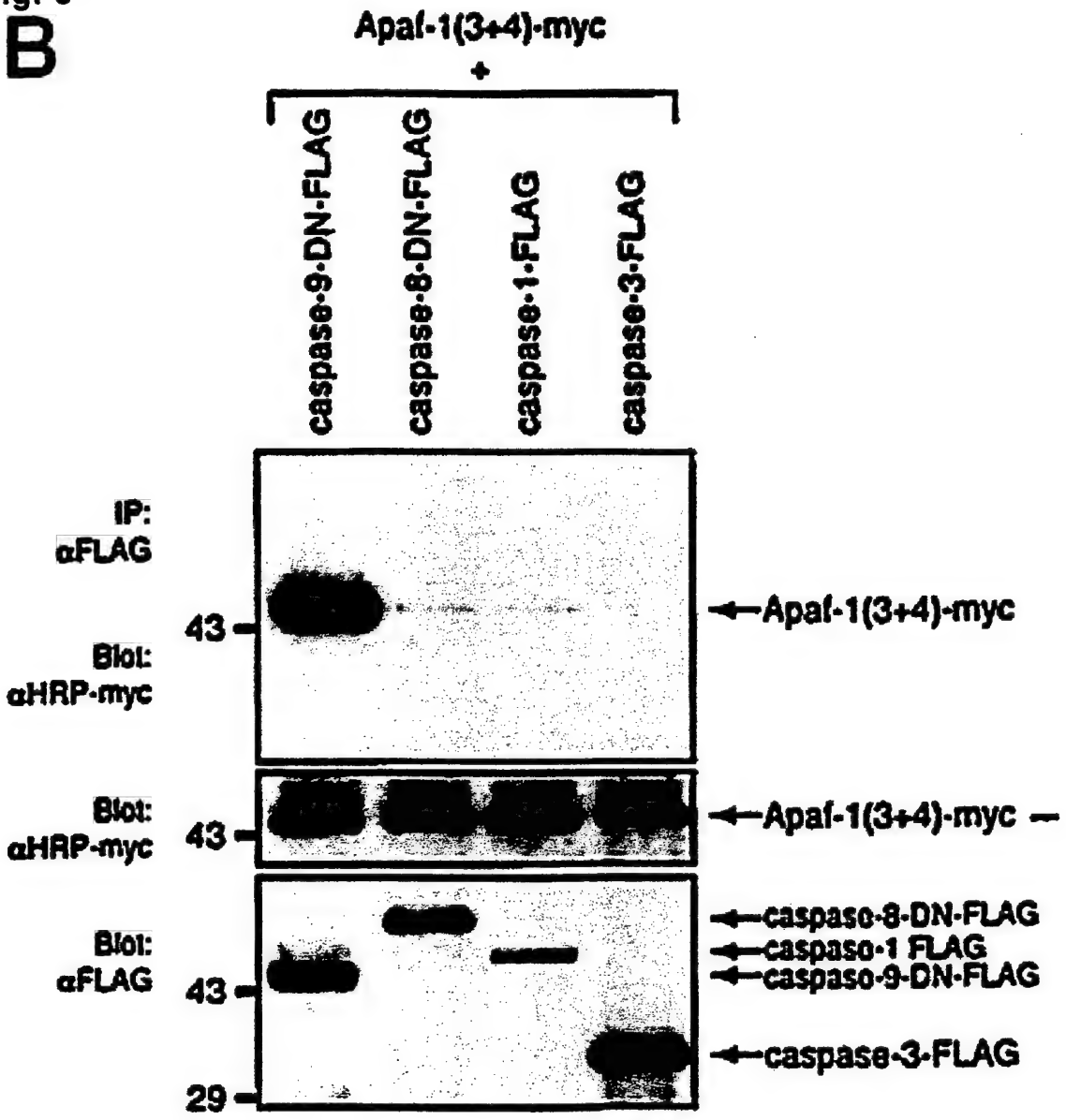
Fig. 5

A



Both Caspase-9 and Bcl-X_L co-immunoprecipitate with Apaf-1.
A) Apaf-1 binds Caspase-9 and Bcl-X_L. 293 cells were transfected with indicated expression constructs. After 36-40 hrs , extracts were prepared and immunoprecipitated (IP) with anti-Flag M_2 affinity gel. The IP's as well a the cell lysates were analyzed by western blotting with the indicated antibodies.

Fig. 5



B) Caspase-9, but not other caspases , interacts with Apaf-1. 293 cells were transfected with Apaf-1(3+4) (amino acids 1-412) and the indicated caspase constructs and analyzed as in A. The middle and bottom panels show the expression of the individual proteins.

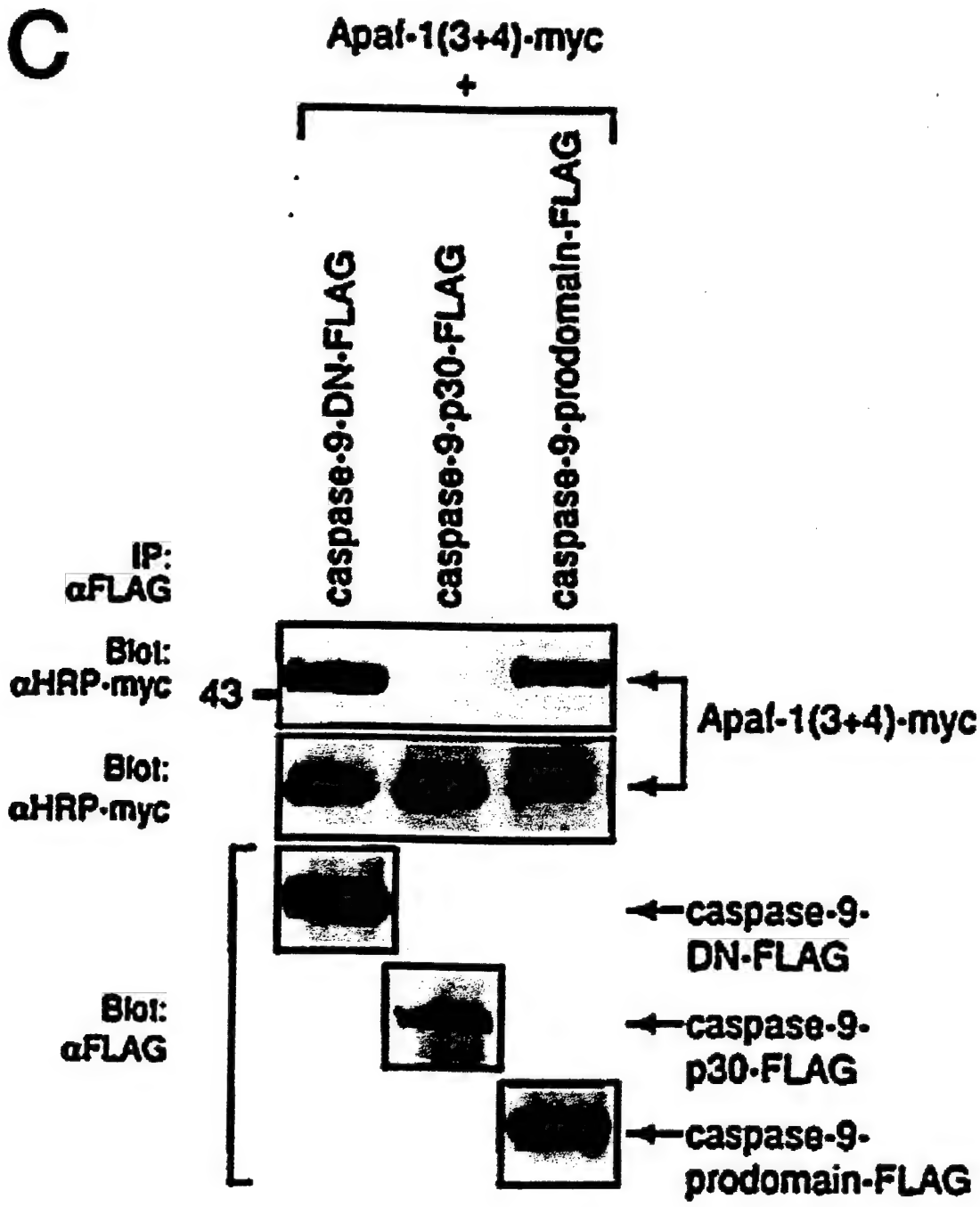
Upon expression in 293 cells both Bcl-X_L and Caspase-9 co-immunoprecipitated with Apaf-1 (Fig.5 A). Caspase-9 DN is a mutant form where the active site cysteine has been mutated to serine. This mutant was used in these experiments to prevent the cells from undergoing apoptosis during the transient expression experiments. Full length, N terminal FLAG-tagged, APAF-1 co-immunoprecipitated with Caspase-9 DN (Fig. 5A lane 1). Co-transfected Bcl-X_L similarly associated with Apaf-1 (lane 2). No decrease in association of either molecule was seen when Caspase-9 DN and Bcl-X_L

where co-transfected with Apaf-1. This is consistent with the formation of a trimolecular complex. The association of Apaf-1 with Caspase-9 is specific as shown in Fig. 5B. Only Caspase-9 co-immunoprecipitated with Apaf-1 construct containing the Ced-3 and Ced-4 homology domains (Apaf-1(3+4) myc). Significantly Caspase-8 and Caspase-1, two other long pro-domain caspases, did not interact with Apaf-1(3+4) (lanes 2 &3). The short pro-domain Caspase-3 also failed to interact with Apaf-1 (lane 4). These results confirm that Caspase-9 is at the apex of the caspase cascade that is triggered by intracellular apoptotic signals as proposed by Li et al. (10).

To identify in more detail the region of Caspase-9 that mediated the interactions co-immunoprecipitation experiments were performed with truncation mutants of Caspase-9.

Fig. 6 (next page)
Caspase-9 associates with Apaf-1 through its CARD containing pro-domain. *C terminal myc tagged Apaf-1 (3+4) (amino acids 1-412) was co-transfected in 293 cells with Caspase-9 mutants carrying a FLAG tag at their C termini. Caspase-9 DN FLAG; full length Caspase-9 with active site cysteine mutated to serine. Caspase-9 p30 FLAG; amino acids 130-416 corresponding to the catalytic domain. Caspase-9 prodomain FLAG; amino acids 1-168 coding for the CARD containing pro-domain. CARD: Caspase recruitment domain. Cleared lysates were immunoprecipitated with FLAG beads and probed with αmyc antibodies. Aliquots of the lysates were also assayed by western blot directly for Apaf-1 expression by αmyc probing (middle panel) and for Caspase-9 mutant expression by αFLAG probing (lower panel).*

Fig. 6


C
Apaf-1(3+4)-myc
+
caspase-9-DN-FLAG
caspase-9-p30-FLAG
caspase-9-prodomain-FLAG
IP:
αFLAG
Blot:
αHRP-myc
43
Apaf-1(3+4)-myc
Blot:
αHRP-myc
caspase-9-
DN-FLAG
Blot:
αFLAG
caspase-9-
p30-FLAG
caspase-9-
prodomain-FLAG

Clearly only full length (Fig. 6 lane 1) and Caspase-9 pro-domain alone (lane 3) were able to associate with Apaf-1 (3+4). The catalytic domain of Caspase-9 (p30) was unable to associate (lane 3). None of these Caspase-9 constructs were able to associate with Apaf-1 (4) constructs containing only the CED-4 homology region (amino acids 86-412) (Fig. 7 lane 1). Consequently, binding of Caspase-9 is due to a homotypic CARD domain interaction.

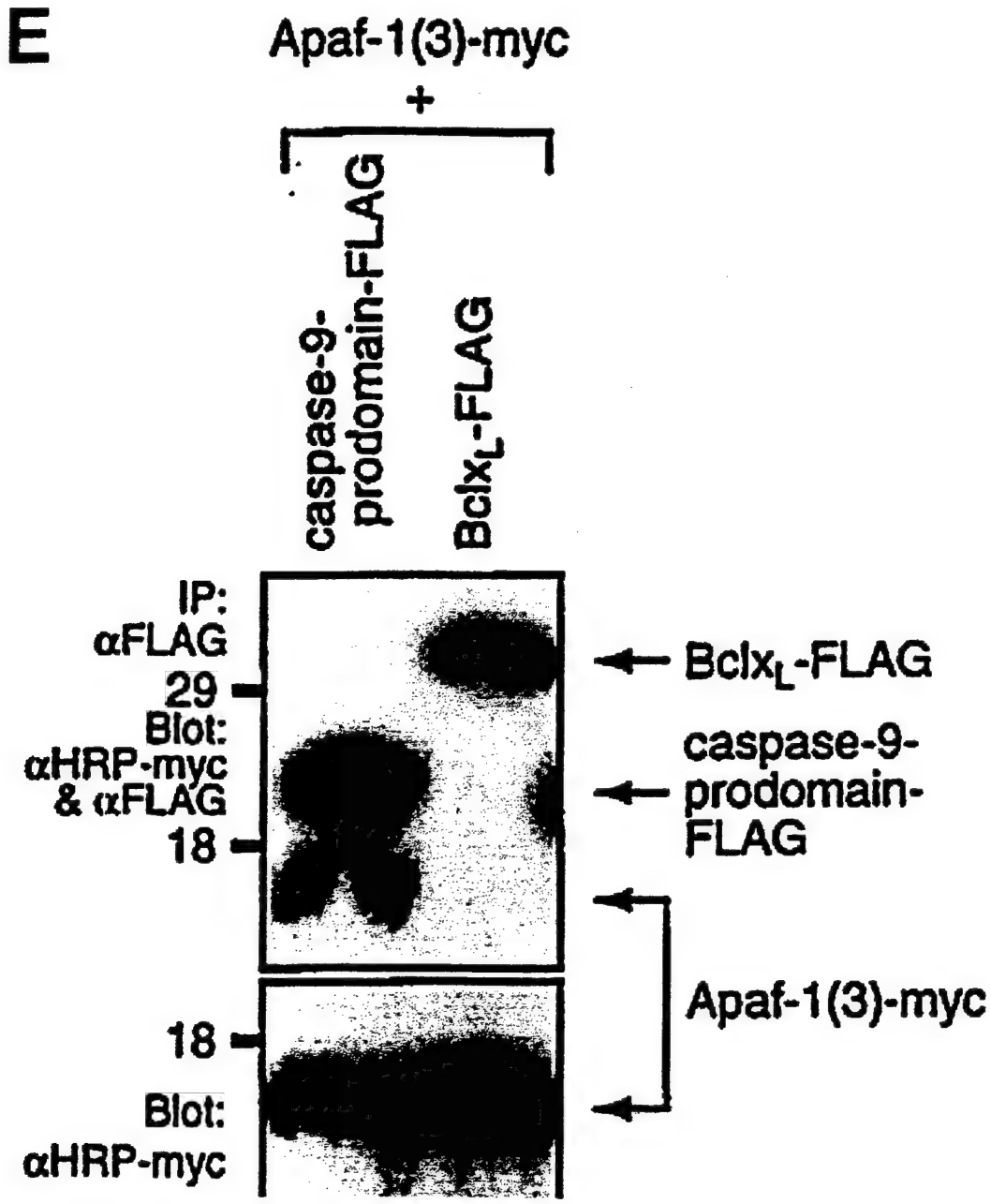
Bcl-X_L on the other hand binds Apaf-1 through the CED-4 homology region (Fig. 7). This domain does not mediate any interactions with Caspase-9. This is further evidence for the formation of a trimeric complex between Apaf-1, Caspase-9, and Bcl-X_L.

We tested the trimolecular complex formation hypothesis directly by showing that Bcl-X_L co-immunoprecipitates with Caspase-9. To proof that this interactions was mediated by endogenous Apaf-1 we co-expressed the CED-3 homology region of Apaf-1. This domain was able to compete with the endogenous Apaf-1, resulting in loss of Bcl-X_L co-precipitation (Fig. 8).

These interactions with Apaf-1 together with the fact that Apaf-1 is the only mammalian homologue of CED-4 implies that Caspase-9 is an non-redundant caspase for multiple apoptotic stimuli. This is corroborated by the fact that expression of the active site mutant of Caspase-9 inhibited death receptor induced cell death as well as Bax and BiK induced apoptosis.

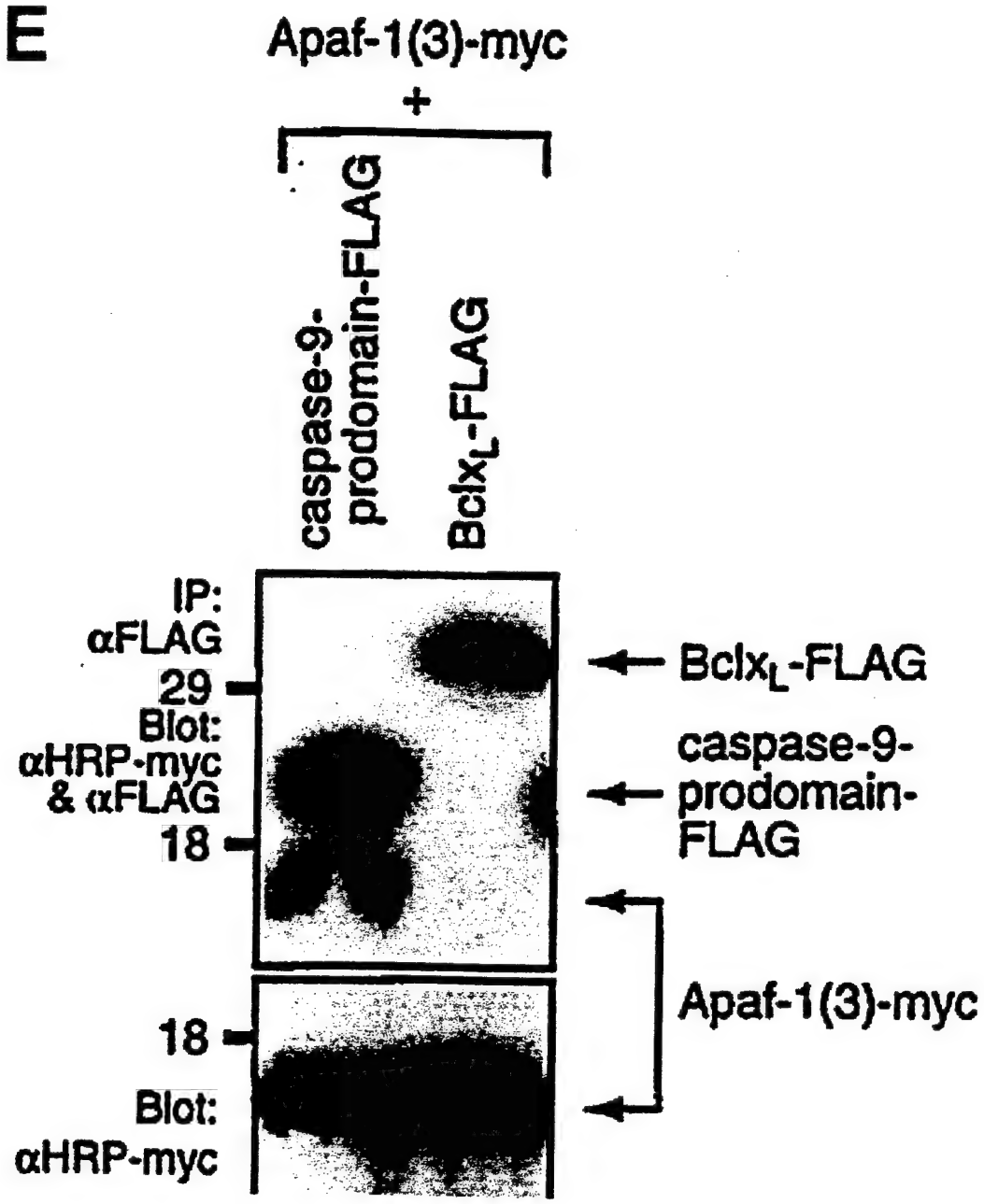
The results of this study were published in Ref (11)

Fig. 7



Bcl-X_L interacts with Apaf-1 via the CED-4 homology domain. *Apaf-1 (4) myc (amino acids 86-412) was co-transfected in 293 cells with Caspase-9-prodomain-FLAG or Bcl-X_L-FALG. The FLAG immunoprecipitates were assayed on a western blot with myc antibodies (upper panel). Expression of the FLAG tagged molecules and Apaf-1 was assayed by western blot analysis with αFLAG or αmyc antibodies respectively (middle and lower panels).*

Fig. 8



Caspase-9 and Bcl-X_L form a ternary complex with Apaf-1. *Caspase-9 associates with Bcl-X_L through and endogenous Apaf-1 like activity. 293 cells were co-transfected with Caspase-9-DN-FLAG, Bcl-X_L-myc and vector (lane 1) or Apaf-1(3), the CARD domain only construct (lane 2). Cell lysates were immunoprecipitated with anti-Flag M_2 affinity gel. Co-expression of the CED-3 homologous region of Apaf-1 disrupts the association of Caspase-9 with Bcl-X_L.*

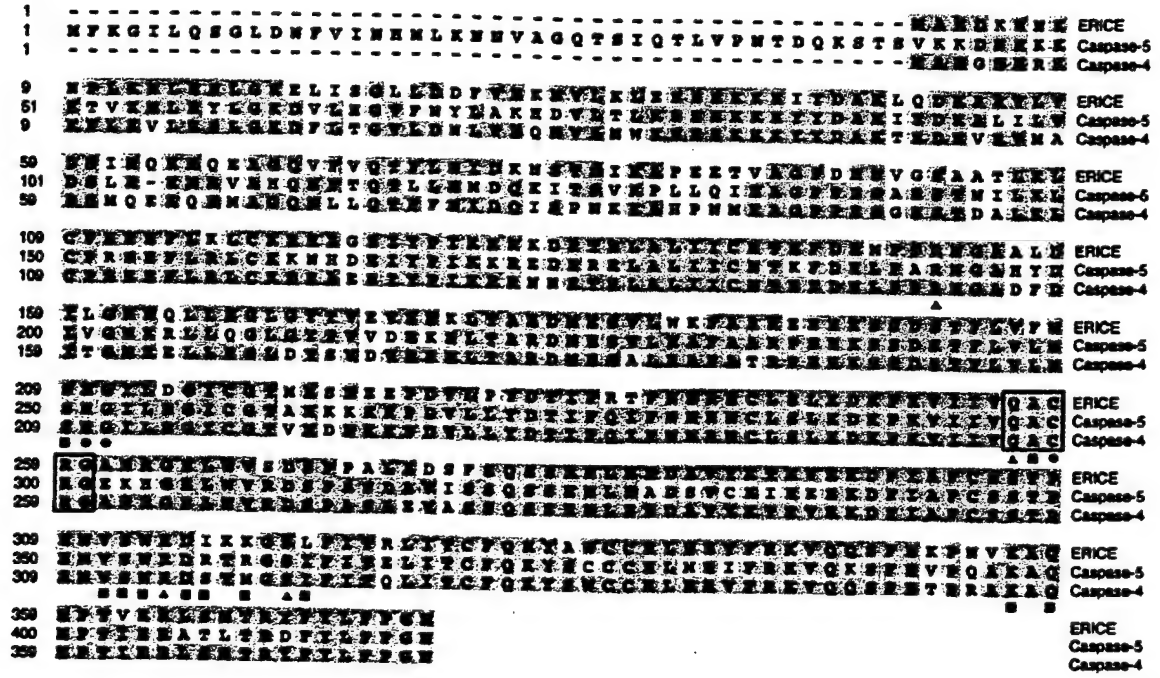
3) Discovery of new caspases

We took advantage of the wealth of sequencing data that is rapidly accumulating in public and proprietary databases to search for molecules with homology to the caspase family of proteases.

ERICE (Caspase-13) has significant identities to Caspase-4 and 5, which are all members of the ICE sub-family of caspases. Despite the high conservation in the primary structure, ERICE is predicted to have different substrate specificity as some of the amino acid residues contacting the P4 substrate site are not conserved (Fig. 9).

Fig. 9

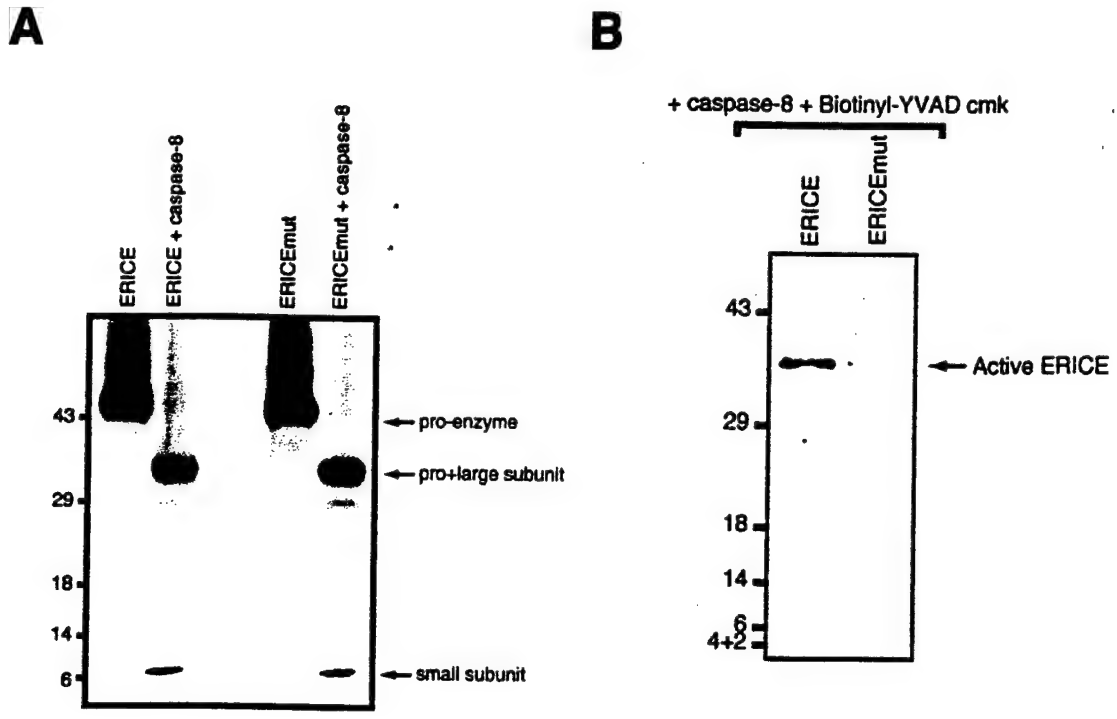
B



Primary structure of ERICE. *Alignment of ERICE with the other members of the ICE subfamily. The conserved pentapeptide is boxed. Based on the x-ray structure of ICE, residues involved in catalysis are indicated by filled circles; filled triangles represent residues that make up the binding pocket for the carboxylate side chain of the P_1 Asp; filled squares indicate residues adjacent to the P_2-P_4 amino acids.*

High levels of ERICE expression is observed in peripheral lymphoid organs, placenta and the lung. Overexpression of ERICE induces apoptosis in 293 as well as MCF7 cells (not shown).

Fig. 10



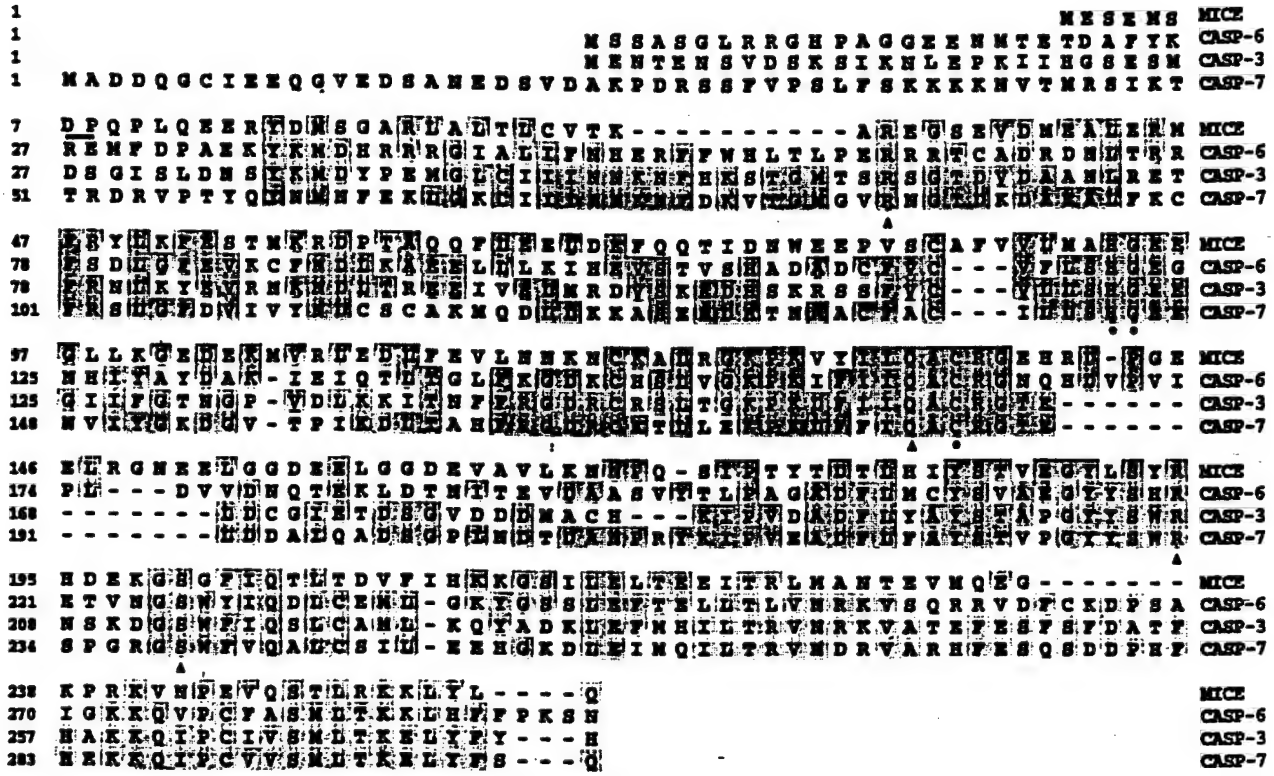
ERICE cleavage and activation by Caspase-8. *Panel A; ERICE wild type or catalytically inactive ERICE (C258S) were in vitro transcription translated and incubated with recombinant active Caspase-8. The input and cleavage products were analyzed by SDS-PAGE and fluorography. Panel B; The Caspase-8 cleaved products were incubated with biotinylated YVAD. The biotinylated complexes were precipitated with streptavidin beads and analyzed by SDS-PAGE and fluorography.*

ERICE may be involved in Caspase-8 mediated apoptosis as it is cleaved by Caspases-8 *in vitro* (Fig. 10 A). We were also able to show that the processed enzyme is active as indicated by its ability to bind the biotinylated caspase substrate YVAD. This is in contrast to Caspase-1 (ICE) which is not activated by Caspase-8. Also, Granzyme B, which cleaves and activates most other caspases, cleaved but did not activate ERICE (not shown).

These results were published in Ref. (12)

Using a similar homology search using the public database we identified a mouse caspase. It is referred to as MICE, for mini ICE, because of its complete lack of a prodomain.

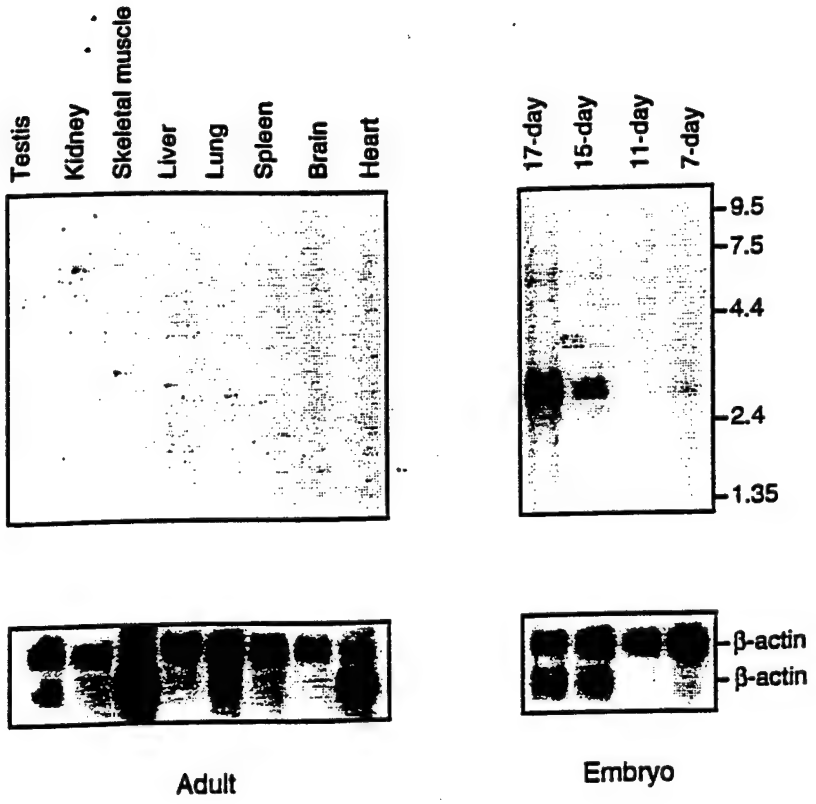
Fig. 11



Sequence alignment of MICE with known short prodomain caspases. *Sequence alignment of MICE and three known short prodomain caspases. Filled circles indicate residues involved in catalysis and triangles identifies residues that form the binding pocket for the carboxylate side chain of P1 Asp. The putative cleavage sites between prodomain and p20 are underlined.*

MICE is only expressed in during mouse development and its mRNA is not detectable in any adult tissue. The unique structure and restricted expression indicates a role for MICE in tissue remodeling during development.

Fig. 12



Tissue distribution of MICE. *Murine adult multiple and embryo poly (A) + Northern blots (Clonetech) were probed with ^{32}P-labeled MICE cDNA (upper panels). The filters were reprobed with β-actin to demonstrate similar loading.*

We are currently analyzing the expression pattern at higher resolution by in situ hybridizations. Mice, renamed caspase-14, is unique among its family members. Its lack of a prodomain suggests that it is not synthesized as an inactive zymogen and that its function is controlled on a transcriptional level. Therefore, studying the transcriptional regulation of MICE would shed light on a novel mechanism for controlling apoptosis.

4) Generation of monoclonal antibodies towards long pro-domain caspases.

As outlined in last years revised SOW we initiated a project to produce monoclonal antibodies against the large pro-domain caspases. The approach was to use the same antigenic peptides that had been used in rabbits to produce western blotting poly-clonal antibodies. With all the peptides injected we obtained mouse tail bleeds that specifically detected the antigenic protein on western blots of transfected 293 cell lysates. A good number of clones were identified that proofed positive by ELISA using plates coated with the antigenic peptide. Unfortunately none of these hybridoma supernatants yielded an antibody with any specificity on westerns.

I have decided not to repeat this effort as commercial antibodies towards large pro-domain caspases become available. We are in the process of testing these antibodies.

5) Identification of new molecules containing death effector domains.

We are currently analyzing clones that were obtained by a two hybrid screen using the pro-domain of I-FLICE as bait. This domain contains DED domains. DED domains are homotypic interactions domains and therefore a two hybrid screen is predicted to isolate molecules containing this domain. All DED containing molecules discovered up to date have low to undetectable expression in neuronal systems. Our working hypothesis is that yet to be discovered homologues function in neuronal systems. Several putative positive clones have been identified (insert size 1.6 kb and several at ~3kb). We are testing the specificity of the interactions in yeast. The sequence information obtained so far indicates that these genes have not been described previously.

Before deciding to analyze these genes in mammalian systems we will test them for specific interaction in yeast exhaustively.

We also take advantage of the large amount of sequences being deposited in the public databank. We update our DED homology searches of the public EST database on a bi-weekly basis. Up to date no new molecules have been identified.

6) Development of a cell culture system for neuronal apoptosis.

We initially investigated the neuroblastoma cell line SH-SY5Y. This cell line has been used as a model for hyperglycemia induced apoptosis (13). Our goal was to investigate what parts of the apoptotic machinery were involved in this paradigm. Stable cell lines were generated expressing vFLIP (E8), DN Caspase-9, and P35. Despite good expression levels none of the clones were protected against 300 mM mannitol induced cell death. P35 lines showed good protection from etoposide induced apoptotsis and E8 cells were partially protected. Given these results it is questionable if mannitol treatment of SH-SY5Y cells is a valid model for apoptosis.

Towards our goal of developing a cell culture system for neuronal apoptosis we are characterizing a panel of neuroblastoma cell lines. We have identified one line SK-N-BE (2) that does not express NGF receptors (Both, TrkA and p75). We have made ecdysone inducible hp75 constructs and are in the process of establishing stable lines. The goal is to set up a system where p75 signaling, particularly apoptotic signaling, can be measured independently of TrkA.

Conclusion

We have made further progress characterizing the FAS and TNFR-1 proximal events that control the activation of the apical Caspase-8.

First, we employed an in vivo cross-linking strategy to show that Caspase-8 can activate itself if aggregated to high local concentration by FADD. We were also able to show that the zymogen of Caspase-8 has low but significant proteolytic activity, which is consistent with the proximity induced activation model.

Secondly, we discovered I-FLICE, a new molecule homologous to Caspase-8 that may be involved in the control of the activation of this caspase. I-FLICE does not have an active site cysteine and is a negative regulator of FAS and p55 induced apoptosis. The two death effector domains in the N-terminus of the molecule are functional interaction domains as it binds to Caspase-8.

Further, we started looking into the activation of other apical caspases, mainly Caspase-9. The dominant negative form of Caspase-9 inhibits a broad range of apoptotic stimuli, including BAX overexpression. This implicates Caspase-9 to be at the apex of the proteolytic cascade induced by intracellular stimuli. The importance of Caspase-9 was further highlighted by the recent discovery that it is associated with Apaf-1, the human CED-4 homologue. We investigated if the triad between the pro-apoptotic genes ced-3, ced-4 and the anti-apoptotic gene ced-9 found in *C. elegans* is also operative in mammalian cells. Indeed, Bcl-x_L interacted with Caspase-9 via Apaf-1. The caspase, Apaf-1 interaction was mediated by their CARD domains while Apaf-1 interacted with Bcl-x_L via its CED-4 homology region. In addition, we showed that Bax a pro-apoptotic family member could compete with Apaf-1 for Bcl-x_L binding .This complex therefore could be the basis for understanding the mechanism of pro- and anti-apoptotic Bcl-2 family members.

We added two new caspases to the growing family of apoptotic proteases. ERICE belongs to the subgroup of ICE-caspases. Caspase-8 cleaves and activates ERICE in contrast to ICE.

The distinguishing mark for MICE is the complete lack of a pro-domain. MICE is only expressed in the developing embryo and not in any adult tissue. This strongly suggests that MICE is a unique caspase required during development and that its activity is controlled on the transcriptional level and not through zymogen activation.

The ongoing projects include characterization of antibodies towards the long pro-domain caspases. The goal is to obtain reagents able to detect the endogenous proteins in different tissues and follow their activation upon exposure to different apoptotic stimuli. Massive apoptosis is observed during the development of the nervous system but very little is known about the signaling events and effector molecules. We are in the process of setting up cell culture models to study neuronal apoptosis and to test new molecules isolated by two hybrid screens from neuronal libraries.

Publications supported by this Grant:

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Hu S, Vincenz C, Ni J, Gentz R, Dixit VM: **I-FLICE, a novel inhibitor of tumor necrosis factor receptor-1 and CD-95 induced apoptosis.** *J Biol Chem* 1997, **272**:17255-57.

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Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 28, Issue of July 11, pp. 17255–17257, 1997
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Printed in U.S.A.

I-FLICE, a Novel Inhibitor of Tumor Necrosis Factor Receptor-1- and CD-95-induced Apoptosis*

(Received for publication, May 6, 1997, and in revised form, May 19, 1997)

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The pivotal discovery that the death proteases caspase 8 (FLICE) and caspase 10 (Mch4/FLICE2) are recruited to the CD-95 and tumor necrosis factor receptor-1 signaling complexes suggested a mechanism used by these cytotoxic receptors to initiate apoptosis. In this report, we describe the cloning and characterization of I-FLICE, a novel inhibitor of tumor necrosis factor receptor-1- and CD-95-induced apoptosis. The overall architecture of I-FLICE is strikingly similar to that of FLICE and Mch4/FLICE2. However, I-FLICE lacks both a catalytic active site and residues that form the substrate binding pocket, in keeping with its dominant negative inhibitory function. I-FLICE is the first example of a catalytically inert caspase that can inhibit apoptosis.

The cell death machinery is conserved throughout evolution and is composed of activators, inhibitors, and effectors (1). The effector arm of the cell death pathway is composed of a rapidly growing family of cysteine aspartate-specific proteases termed caspases (2). As implied by the name, these cysteine proteases cleave substrates following an aspartate residue (2, 3). Caspases are normally present as single polypeptide zymogens and contain an N-terminal prodomain and large and small catalytic subunits (4–6). The two-chain active enzyme (composed of the large and small subunits) is obtained following proteolytic processing at internal Asp residues (4–6). As such, caspases are capable of activating each other in a manner analogous to zymogen activation that is observed in the coagulation cascade (7). The identification of FLICE and Mch4/FLICE2 as receptor-associated caspases suggested a surprisingly direct mechanism for activation of the death pathway by the cytotoxic receptors CD-95 and TNFR-1[1] (7–10). Upon activation, both receptors use their death domains to bind the corresponding domain in the adaptor molecule FADD (Fas-associated death domain protein) (8–10). Dominant negative versions of FADD that lack the N-terminal segment but still retain the death domain potently inhibit both CD-95- and TNFR-1-induced apoptosis (11, 12). Given the importance of the N-terminal segment in engaging the death pathway, it has been termed the death effector domain (DED) (11).

Remarkably, the DED is present within the prodomain of FLICE and Mch4/FLICE2, and mutagenesis studies suggest that a homophilic interaction between the DED of FADD and the corresponding domain in FLICE or Mch4/FLICE2 is responsible for the recruitment of these proteases to the CD-95 and TNFR-1 signaling complexes (8–11). Taken together, these data are consistent with FLICE and Mch4/FLICE2 being apical enzymes that initiate precipitous proteolytic processing of downstream caspases resulting in apoptosis (7, 13–15). A number of viral gene products antagonize CD-95- and TNFR-1-mediated killing as a means to persist in the infected host (16). The poxvirus-encoded serpin CrmA and baculovirus gene product p35 are direct caspase inhibitors (3). In contrast, the molluscum contagiosum virus protein MC159 and the equine herpesvirus protein E8 encode DED-containing decoy molecules that bind to either FADD (MC159) or FLICE (E8) and disrupt assembly of the receptor signaling complex, thereby abrogating the death signal (17–19). The existence of these viral inhibitors has raised the question of whether functionally equivalent molecules are encoded in the mammalian genome.

Here, we report the cloning and characterization of a novel mammalian inhibitor designated I-FLICE (for inhibitor of –FLICE), a catalytically inactive structural homologue of FLICE and Mch4/FLICE2 that inhibits both TNFR-1- and CD-95-induced apoptosis. This is the first example of a naturally occurring catalytically inactive caspase that can act as a dominant negative inhibitor of apoptosis.

MATERIALS AND METHODS

Cell Lines and Expression Vectors—Human embryonic kidney 293, 293T, and 293-EBNA cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, nonessential amino acids, L-glutamine, and penicillin/streptomycin. Expression constructs were made in pcDNA3 or pcDNA3.1/MycHisA (Invitrogen) using standard recombinant methodologies (20).

Cloning of I-FLICE—cDNAs corresponding to the partial open reading frame of I-FLICE were identified as sequences homologous to FLICE and Mch4/FLICE2 on searching the Human Genome Sciences data base using established expressed sequence tag methods (21, 22). Full-length cDNAs were obtained by screening a random-primed human umbilical vein endothelial cell cDNA library constructed in the pcDNA1 vector (Invitrogen). The sequence of I-FLICE was confirmed by sequencing plasmid DNA template on both strands by the dideoxy chain termination method employing modified T7 DNA polymerase (Sequenase, U. S. Biochemical Corp.)

Northern Blotting—Human multiple tissue and human cancer cell line poly(A)$^+$ RNA blots were obtained from CLONTECH and processed according to the manufacturer's instructions.

Transfection, Coimmunoprecipitation, and Western Analysis—Transient transfections of 293T cells were performed as described previously (23). Cells were harvested 40 h following transfection, immunoprecipitated with the indicated antibodies, and analyzed by immunoblotting.

Cell Death Assay—Human embryonic kidney 293 (for TNFR-1 killing) or 293 EBNA cells (for CD-95 killing) were transiently transfected with 0.1 μg of the reporter plasmid pCMV β-galactosidase plus 0.5 μg of test plasmid in the presence or the absence of 2.0 μg of inhibitory plasmids. 22–24 h following transfection, cells were fixed in 0.5% glutaraldehyde and stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside. Percentage of apoptotic cells was determined by calculating the fraction of membrane blebbed blue cells as a function of total blue

* This work was supported by National Institutes of Health Grants ES08111 and AG13671. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: TNFR-1, tumor necrosis factor receptor 1; FADD, Fas-associated death domain protein; FLICE, FADD-like interleukin-1β-converting enzyme; FLICE2, FADD-like ICE2; DED, death effector domain.

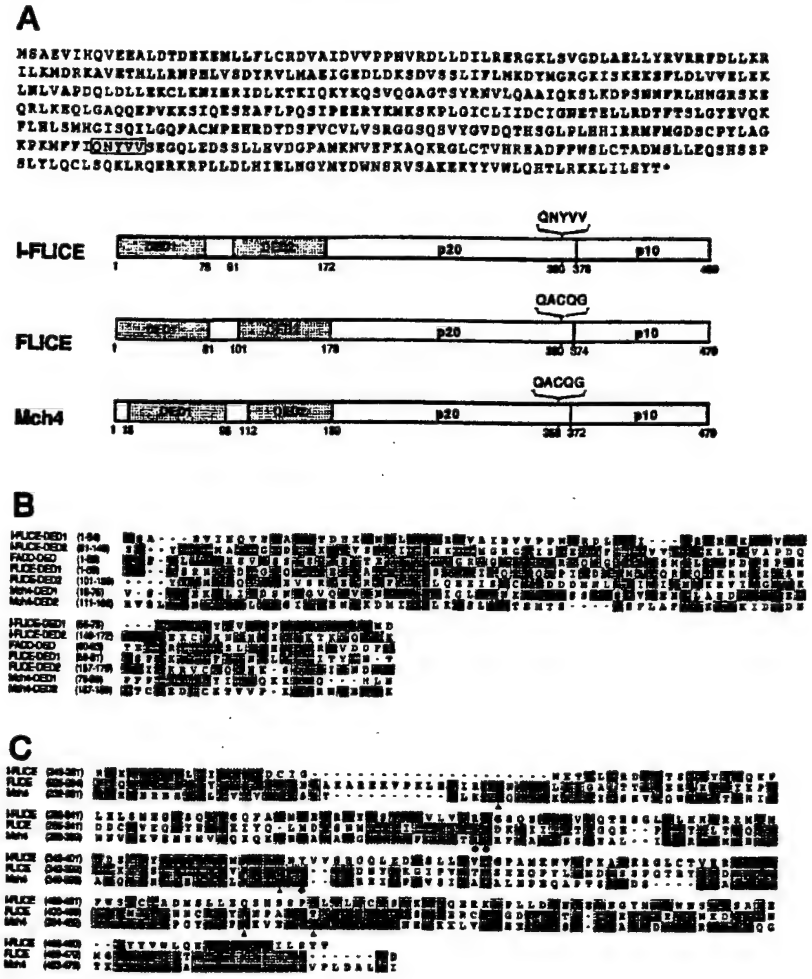

FIG. 1. **Sequence analysis of I-FLICE.** *A*, deduced amino acid sequence of I-FLICE. The pentapeptide corresponding to the conserved active site (QACR(Q)G) of other caspases is *boxed*. Shown underneath are schematic models of I-FLICE, FLICE, and Mch4. The N-terminal portion of each molecule contains two DED-like domains (*shaded boxes*), and the C-terminal portion contains both large and small catalytic subunits (p20/p10). The pentapeptide QNYVV of I-FLICE and the corresponding motifs in FLICE and Mch4 are indicated. *B*, the two DED motifs of I-FLICE were aligned with DED motifs present in FADD, FLICE, and Mch4. *C*, the C-terminal region of I-FLICE was aligned with the catalytic domains of FLICE and Mch4. ● symbols indicate residues involved in catalysis, and ▲ symbols identify residues that form the binding pocket for the carboxylate side chain of P1 Asp.

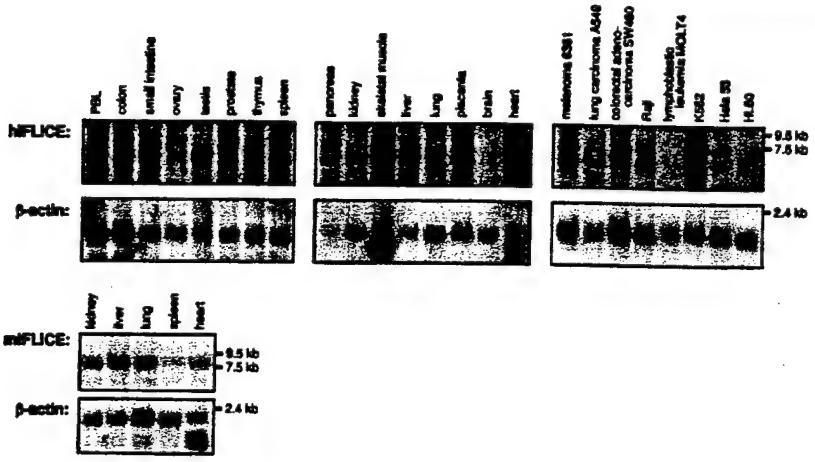

FIG. 2. **Tissue distribution of I-FLICE.** Human adult and cell lines poly(A)$^+$ Northern blots were probed with ^{32}P-labeled human I-FLICE probe corresponding to codons 215–331. *PBL*, peripheral blood leukocyte. The mouse tissue was Northern probed with a 200-base pair mouse I-FLICE probe complementary to the 5′ end.

cells. All assays were evaluated in duplicate, and the mean and standard deviations were calculated.

RESULTS AND DISCUSSION

Sequence of I-FLICE—Sequence analysis of a full-length cDNA revealed a 1443-base pair open reading frame that encoded a novel protein with a predicted molecular mass of 55.3 kDa (Fig. 1*A*). Given that the protein had striking homology to FLICE and Mch4/FLICE2 but lacked an active site, making it a potential dominant negative inhibitor, it was designated I-FLICE (for inhibitor of FLICE).

The architecture of I-FLICE was strikingly similar to that of FLICE and Mch4/FLICE2, including two N-terminal DED-like tandem repeats and a region that resembled the caspase catalytic domain (Fig. 1, *B* and *C*). Overall, I-FLICE displayed 19 and 18% identity (32 and 28% similarity) to FLICE and Mch4, respectively. Importantly, I-FLICE did not contain the catalytic cysteine that is normally embedded in the conserved pentapeptide QACRG or QACQG motif present in all known caspases. Rather, the pentapeptide sequence was QNYVV. In addition, based on the x-ray crystal structure of caspase-1 (and caspase-3), amino acid residues His^{237} (His^{121}), Gly^{238} (Gly^{122}), and Cys^{285} (Cys^{163}) are involved in catalysis, whereas residues Arg^{179} (Arg^{64}), Gln^{283} (Gln^{161}), Arg^{341} (Arg^{207}), and Ser^{347} (Ser^{213}) form a binding pocket for the carboxylate side chain of the P1 aspartic acid (4–6). These seven residues are conserved in all caspases, but only three of them (Gly, Gln, and Ser as indicated in Fig. 1*C*) are found in I-FLICE. Given this lack of conservation of key residues involved in catalysis and substrate

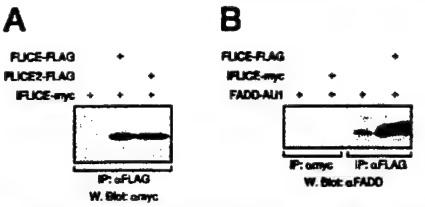

FIG. 3. **I-FLICE binds FLICE and FLICE2 but not FADD.** 293T cells were co-transfected with indicated plasmids. Following transfection, cells lysates were immunoprecipitated (*IP*) and immunoblotted with the respective epitope tag antibodies. *W. Blot*, Western blot.

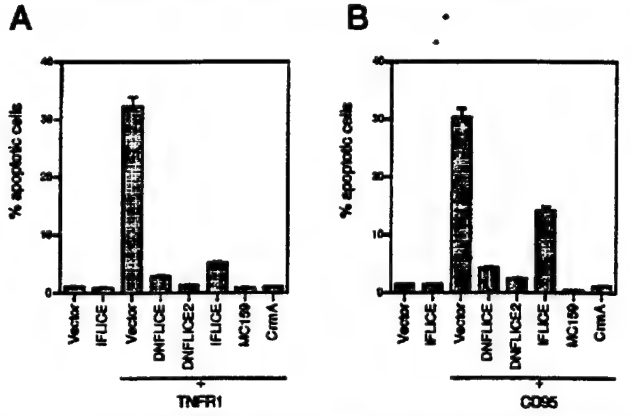

FIG. 4. **I-FLICE inhibits TNFR-1- and CD95-induced apoptosis.** Overexpression of I-FLICE attenuated TNFR-1-induced (*A*) and CD-95-induced (*B*) cell death. 293 (*A*) or 293-EBNA (*B*) cells were co-transfected with the indicated plasmids together with the reporter construct pCMV β-galactosidase. The data shown are the percentages of blebbing blue cells as a function of total number of blue cells counted.

binding, it can be concluded that I-FLICE is not a cysteine protease and is incapable of binding Asp at the P1 position. Interestingly, the DED domain of I-FLICE was more related to the corresponding domains present in the viral DED-containing inhibitors, sharing 32, 30, and 29% identity (52, 49, and 39% similarity) to K13, MC159, and E8, but only 23 and 18% identity (44 and 30% similarity) to FLICE and Mch4, respectively (17, 19).

Tissue Distribution of I-FLICE—Human tissue and cell line RNA blots were probed with a ^{32}P-labeled cDNA specific for I-FLICE. Two transcripts (7.5 and 6 kilobases) were observed, possibly due to differential polyadenylation (Fig. 2). The tissue distribution was similar to that of FLICE and Mch4/FLICE2 (8–10, 15), being expressed in most tissues and cell lines examined except for the brain and the lymphoblastic leukemia line MOLT4. In particular, I-FLICE expression was evident in peripheral blood leukocytes, spleen, placenta, and heart. In mouse tissues the only predominant mRNA species is the 7.5-kilobase form.

I-FLICE Associates with FLICE and FLICE2—Previous studies have shown that the DED domain is a protein interaction motif that mediates the binding of the adaptor molecule FADD to the effector proteases FLICE and Mch4/FLICE2 (8, 10). Given the striking structural similarity, we asked if I-FLICE interacted with either FADD or other FLICE-like caspases. Co-immunoprecipitation analysis clearly revealed the ability of I-FLICE to bind FLICE and Mch4/FLICE2 (Fig. 3*A*), but not FADD (Fig. 3*B*). In this respect, I-FLICE resembles the viral DED-containing molecule E8 in that it binds FLICE but not FADD (17, 18). Because there was no association between I-FLICE and FADD, I-FLICE was not recruited to the CD-95 or TNFR-1 signaling complex as evidenced by its inability to co-precipitate with these receptors (data not shown).

I-FLICE Inhibits TNFR-1 and CD-95-induced Apoptosis—Given the ability of catalytically inactive I-FLICE to complex with FLICE-like caspases, we reasoned that I-FLICE may be acting as a dominant negative inhibitor because the active form of all caspases is a tetramer derived from the processing of two zymogen forms to a four-chain assembly (4–6). It follows that a catalytically inert zymogen, such as I-FLICE, would be processed to inactive subunits that would result in the generation of a nonfunctional tetrameric protease. Although it is presently conjecture, this putative mechanism does predict that I-FLICE should inhibit TNFR-1 and CD-95-induced apoptosis where FLICE-like caspases play an initiating role. Consistent with the prediction, overexpression of I-FLICE resulted in substantial inhibition of TNFR-1-induced cell death comparable with previously characterized inhibitors including CrmA, MC159, dominant negative FLICE, and dominant negative Mch4/FLICE2 (Fig. 4*A*). However, under the present experimental conditions, I-FLICE appeared to be a less potent inhibitor of CD-95-induced cell death, possibly reflecting the more potent death signal that emanates from this receptor.

In summary, our studies have identified a catalytically inactive member of the caspase family that can serve as a dominant negative inhibitor of CD-95- and TNFR-1-induced cell death by binding and antagonizing the apical FLICE-like caspases. Additional studies will be necessary to work out in detail the exact nature of the inhibitory mechanism.

Acknowledgments—We thank Justin McCarthy, Arul Chinnaiyan, Marta Muzio, and Karen O'Rourke for providing reagents and helpful discussions and Ian Jones for help in preparing the figures.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 25, Issue of June 19, pp. 15702–15707, 1998
Printed in U.S.A.
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ERICE, a Novel FLICE-activatable Caspase*

(Received for publication, December 12, 1997, and April 2, 1998)

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Programmed cell death, or apoptosis, is a process of fundamental importance to cellular homeostasis in metazoan organisms (Ellis, R. E., Yuan, J., and Horvitz, H. R. (1991) *Annu. Rev. Cell Biol.* 7, 663–698). The caspase family of mammalian proteases, related to the nematode death protein CED-3, plays a crucial role in apoptosis and inflammation. We report here the isolation and characterization of a new caspase, tentatively termed ERICE (Evolutionarily Related Interleukin-1β Converting Enzyme). Based on phylogenetic analysis, ERICE (caspase-13) is a member of the ICE subfamily of caspases which includes caspase-1 (ICE), caspase-4 (ICE_{rel}-II, TX, ICH-2), and caspase-5 (ICE_{rel}-III, TY). Overexpression of ERICE induces apoptosis of 293 human embryonic kidney cells and MCF7 breast carcinoma cells. Like other members of the subfamily, ERICE is not activated by the serine protease granzyme B, a caspase-activating component of cytotoxic T cell granules. Therefore, ERICE most likely does not play a role in granzyme B-induced cell death. ERICE, however, was activated by caspase-8 (FLICE, MACH, Mch-5), the apical caspase activated upon engagement of death receptors belonging to the tumor necrosis factor family. This is consistent with a potential role for ERICE in this receptor-initiated death pathway.

Apoptosis, or programmed cell death, is an evolutionarily conserved process central to the normal development and homeostasis of multicellular organisms (1). Genetic analysis of the nematode *Caenorhabditis elegans* has revealed three core components of the death pathway, *ced-3*, *ced-4*, and *ced-9* (2). *ced-3* and *ced-4* are death genes, and mutations in either attenuate the elimination of cells that normally die during worm development. These central players of cell death in the nematode are conserved in vertebrates. The CED-3 molecule was found to be homologous to the mammalian cysteine protease ICE[1] (caspase-1) (3–6). Since that time, numerous mammalian counterparts to CED-3 have been discovered and comprise an emerging family of proteases recently termed caspases to denote the conserved cysteine residue in the active site and the ability of the proteases to cleave following aspartate residues (7).

Currently, the caspase family consists of 12 members, many of which have a proven role in inflammation or apoptosis. Members include ICE (caspase-1) (3, 4), ICH-1 (caspase-2) (8), CPP32, apopain, Yama (caspase-3) (9–11), ICE_{rel}-II, TX, ICH-2 (caspase-4) (12–14), ICE_{rel}-III, TY (caspase-5) (12), Mch2 (caspase-6) (15), Mch3, ICE-LAP3, CMH-1 (caspase-7) (16–18), FLICE, MACH, Mch5 (caspase-8) (19–21), ICE-LAP6, Mch6 (caspase-9) (22–24), and Mch4 (caspase-10) (21, 25). Two more murine caspases with no known human counterparts have been described mICH-3, mCASP-11 (caspase-11) (26, 27) and mICH-4, mCASP-12 (caspase-12) (27, 28). All members of the caspase family share a number of amino acid residues crucial for substrate binding and catalysis (29, 30). For caspase-1, these residues include Cys^{285} and His^{237}, which hydrogen bond with the thiohemiacetal of the enzyme-substrate complex, and Gly^{238}, which forms the oxyanion hole that stabilizes the oxyanion of the reaction intermediates. Arg^{179}, Gln^{283}, Arg^{341}, and Ser^{347} form the binding pocket for the carboxylate side chain of the P_1-aspartic acid.

Although the mechanism of their protease action is conserved, differences in primary sequences account for differential substrate specificity. The caspase family has been divided into three groups based upon substrate specificity using a positional scanning substrate combinatorial library (31). The most divergent amino acid substitution occurs at substrate position P_4. Caspases-2, -3, and -7, and CED-3 have a preference for the motif DExD↓, and caspases-6, -8, and -9 display a specificity for (I/V/L)ExD↓. Members of these two subfamilies play a prominent role in cell death. In contrast, caspases 1, 4, and 5, which belong to the ICE subfamily and possess a WEHD↓ substrate specificity, appear to play a primary role in cytokine maturation and inflammation. Hydrophobic amino acids are not found in the P_4 position of substrates known to be cleaved during cell death (31).

Caspase inhibitors have been constructed based upon the presumed substrate specificity of the different caspases. The first caspase-1 subfamily specific inhibitor was based upon the tetrapeptide recognition sequence YVAD present in prointerleukin-1β, a natural substrate for caspase-1. Similarly, a tetrapeptide inhibitor was designed around the putative PARP cleavage site, DEVD, to be selective for the proapoptotic caspases (32). Aldehyde derivatives of these inhibitors are reversible and form a thiohemiacetal with the active site cysteine. Chloromethyl, fluoromethyl, acyloxymethyl, diazomethyl, α-pyrazoloxymethyl, and phosphinyloxymethyl ketones function as irreversible inhibitors by covalently forming a thiomethyl ketone to the sulfur atom of the active site cysteine in the large subunit (30). *In vitro* data show Ac-YVAD-aldehyde to be a potent inhibitor of caspase-4 and caspase-1 but ineffective in inhibiting caspase-3 and caspase-7. In contrast, caspase-3

* This work was supported in part by National Institutes of Health Grant R01 AG13671. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Fellow of the Medical Scientist Training Program (MSTP) and supported by MSTP National Institutes of Health Training Grant 5T32 GM07863-16 and by funds from the Department of Pathology.

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[1] The abbreviations used are: ICE, interleukin 1β-converting enzyme; EST, expression sequence tags; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

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and caspase-7 are potently inhibited by Ac-DEVD-CHO (33). Not surprisingly, a number of pathogenic viruses also encode caspase inhibitors as a means of attenuating immune surveillance mechanisms that work by inducing apoptosis of infected cells. For example, the pox virus cytokine response modifier protein CrmA inhibits caspase-1 and -8 most effectively (11, 34), whereas the baculovirus protein p35 inhibits numerous activated caspases (6, 35).

Caspases exist as single polypeptide zymogens composed of a prodomain of variable length plus a large subunit (which contains the catalytic cysteine) and a small subunit (for reviews, see Refs. 36–40). These subunits are flanked by Asp-X sites, suggesting that activation is mediated by an aspartate-specific protease. All caspases require accurate processing at the internal aspartate residues to generate an active dimeric enzyme. Activated members of the caspase family function as the effector arm of the cell death pathway by cleaving a growing number of cellular substrates including lamins (41–44), kinases (45, 46), DNA fragmentation factor (47), keratin-18 (48), FAK (49), as well as other caspases.

Granzyme B, an aspartate-directed serine protease employed by cytotoxic T cells to kill target cells (50), is the only other enzyme known to cleave and activate multiple caspase members involved in apoptosis including caspase-2 (51), -3 (52, 53), -6 (42), -7 (54, 55), -8 (19), -9 (22), and -10 (21). Analysis of the sites of cleavage of caspase-3, -7, and -10 indicates that granzyme B preferentially cleaves at the IXXD sequence found at the COOH-terminal end of the large subunit. Initial cleavage at this site has been shown to be essential for productive processing of caspase zymogens (21, 55, 56). However, granzyme B will not productively process members of the ICE subfamily in keeping with the less prominent role for this subfamily in apoptosis (57).

Caspase-8 is the initiating caspase in the apoptotic cascade that is activated by engagement of death receptors belonging to the tumor necrosis factor receptor family. Caspase-8 contains two NH_2-terminal tandem repeats within the prodomain which are homologous to the death effector domain in the adaptor molecule FADD and allow for its recruitment to the receptor signaling complex. The remainder of the molecule is highly similar to the CED-3 subfamily of caspases (19, 20). In a cell-free system, caspase-8 has been shown to process a variety of proapoptotic caspases; but like granzyme B, it did not activate members of the ICE subfamily (34).

We report here the cloning and characterization of a novel member of the ICE subfamily termed ERICE for Evolutionarily Related ICE (caspase-13). Based upon sequence identity, it is most related to caspase-4 and caspase-5. Overexpression of ERICE in MCF7 and 293 cells induces apoptosis that can be blocked by p35 or CrmA. As expected, mutation of the catalytic cysteine abolishes its proapoptotic activity. In keeping with it being a member of the ICE subfamily, ERICE is not activated by granzyme B. However, unlike other characterized members of the ICE subfamily, ERICE is activated by caspase-8, consistent with a potential role in apoptosis.

MATERIALS AND METHODS

Cloning of ERICE—A data base containing more than 1 million ESTs (expression sequence tags) was generated through the combined efforts of Human Genome Sciences and the Institute for Genomic Research using high throughput automated DNA sequence analysis of randomly selected human cDNA clones (58, 59). Sequence homology comparisons of each EST were performed against the GenBank data base using the BLAST and BLASTN algorithms (60). ESTs having homology to previously identified sequences (probability equal to or less than 0.01) were given a tentative designation based on the identity of the sequence. A specific homology and motif search using the known amino acid and DNA sequence of caspase-4 (12) revealed an EST possessing >50% homology to caspase-4. A full-length clone (HFJAB36) representative of the original EST was obtained by screening a human skin fibroblast cDNA library.

Expression Vectors—Expression constructs encoding full-length ERICE were made with a COOH-terminal hemagglutinin tag. Alteration of the active site cysteine to alanine was accomplished by site-directed mutagenesis employing a four-primer two-step polymerase chain reaction protocol as described previously (61). Alteration of potential aspartate cleavage sites was performed following the QuikChange® site-directed mutagenesis kit (Stratagene). The fidelity of polymerase chain reaction for full-length and mutant expression constructs was confirmed by sequence analysis.

Apoptosis Assay—Cell death assays were performed as described (62).

Northern Blot Analysis—Adult and fetal human multiple tissue Northern blots (CLONTECH) containing 2 μg/lane poly(A)$^+$ RNA were hybridized according to the manufacturer's instructions, using a ^{32}P-labeled *Bam*HI/*Pvu*II 400-base pair restriction fragment containing most of the ERICE prodomain.

Cleavage Assays and Activation—In vitro translated ^{35}S-labeled ERICE and its mutants were treated using either granzyme B or recombinant caspase-8 as described previously (22, 34, 53). To determine whether ERICE was productively processed on exposure to caspase-8 or granzyme B, radiolabeled ERICE was incubated with biotinylated YVAD chloromethyl ketone as described previously (34, 63) and active site-labeled protease precipitated with streptavidin-agarose. Streptavidin beads were washed three times with biotinylation buffer (20 mM Tris, pH 8.0, 0.1% CHAPS, 2 mM dithiothreitol), and bound protease was eluted by boiling in sample buffer, resolved on a 10–20% acrylamide gel, and visualized by autoradiography. The cleavage boundaries were mapped using ERICE mutants in which candidate aspartate residues were changed to alanine. For *in vivo* cleavage studies, mutant ERICE was cotransfected with FLICE or Fas with or without CrmA. Lysates were made with 500 μl of lysis buffer as described previously (19). Immunoblotting was performed with anti-hemagglutinin or anti-FLAG antibodies.

RESULTS AND DISCUSSION

ERICE Is a Novel Member of the Caspase Gene Family—The full-length 2.2-kilobase ERICE cDNA contained an open reading frame of 377 amino acids encoding a protein of predicted molecular mass 43.0 kDa (Fig. 1*A*). A BLAST search of the GenBank protein sequence data base revealed ERICE to possess significant homology to other members of the caspase family, particularly the ICE subfamily, which includes caspase-1, caspase-4, caspase-5, and murine caspases-11 and -12 (Fig. 1*B*). ERICE has 75% sequence identity to caspase-4, 61% sequence identity to caspase-5, and 47% identity to caspase-1. Based on the x-ray crystal structure of ICE (29), several amino acid residues critical for binding and catalysis have been identified. These residues include the catalytic dyad Cys^{285} and His^{237}, and Gly^{238}, which stabilizes the tetrahedral intermediate. Arg^{179}, Gln^{283}, Arg^{341}, and Ser^{347} form the binding pocket for the S_1 subsite. These seven residues are conserved in all caspases thus far characterized including ERICE (Fig. 1*B*). Although the sequence homology is very high between ERICE and caspases-4 and -5, residues that make up the P_2–P_4 binding pocket are not conserved, suggesting a different substrate specificity. The QAC(R/Q/G)G motif conserved in all caspases is conserved in ERICE (Fig. 1*B*). ERICE is the 13th member of the caspase family (Fig. 1*C*) and is tentatively designated caspase-13.

Tissue Distribution of ERICE—Northern blot analysis revealed ERICE to be constitutively expressed mainly in peripheral blood lymphocytes, spleen, and placenta. The transcript size of 1.6 kilobases corresponded to the full-length cDNA obtained from the fibroblast cDNA library (Fig. 2*A*). ERICE was highly expressed in HeLa cells but not in transformed hematopoietic cell lines including Burkitt's lymphoma, Raji cells, or in promyelocytic leukemia HL-60 cells (Fig. 2*B*).

Overexpression of ERICE Induces Apoptosis—To study the functional role of ERICE, we transiently transfected MCF7 breast carcinoma cells and 293 embryonic kidney cells with an

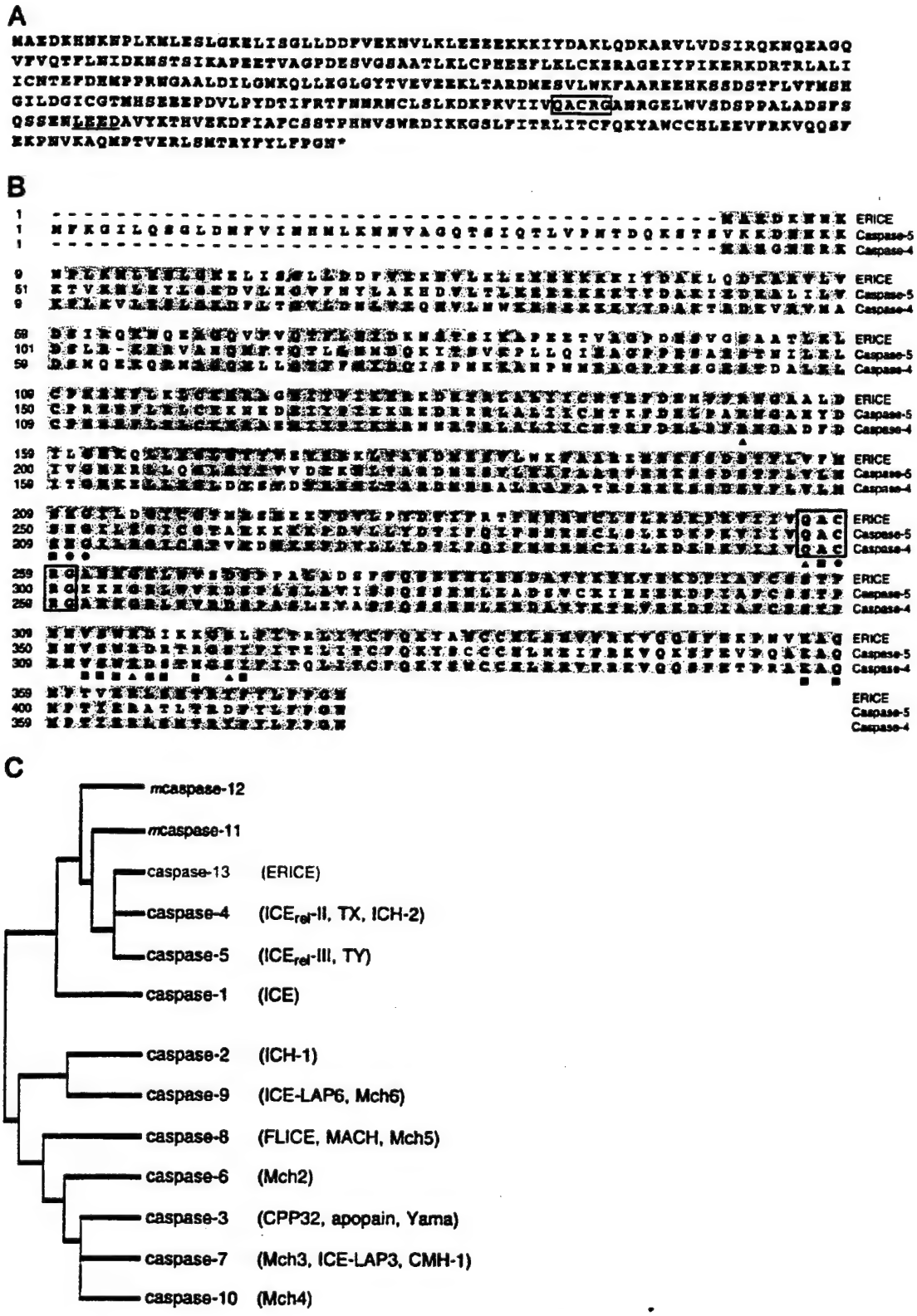


FIG. 1. **Sequence analysis of ERICE.** *Panel A*, predicted amino acid sequence of ERICE. The conserved pentapeptide containing the catalytic cysteine is *boxed*. The caspase-8 cleavage site is *underlined*. *Panel B*, alignment of ERICE with other members of the ICE subfamily. The conserved pentapeptide is *boxed*. Based on the x-ray structure of ICE, residues involved in catalysis are indicated by *filled circles*; *filled triangles* represent residues that make up the binding pocket for the carboxylate side chain of the P_1 Asp; *filled squares* indicate residues adjacent to the P_2–P_4 amino acids. *Panel C*, phylogenetic analysis of the caspase family members showing ERICE (caspase-13). Aliases for the proteases are shown in *parentheses*.

expression vector encoding full-length ERICE and assayed for apoptosis. Like other caspases, ERICE was able to induce cell death (Fig. 3, *A* and *B*). However, unlike caspase-4 and caspase-5, removal of the prodomain was not necessary to induce apoptosis (12). ERICE-transfected MCF7 and 293 cells displayed morphologic alterations typical of adherent cell lines undergoing apoptosis (Fig. 3*C*). To determine whether protease activity was required for inducing apoptosis, the catalytic cysteine was altered to an alanine. Consistent with a requirement for enzymatic activity, the Cys to Ala mutant did not induce

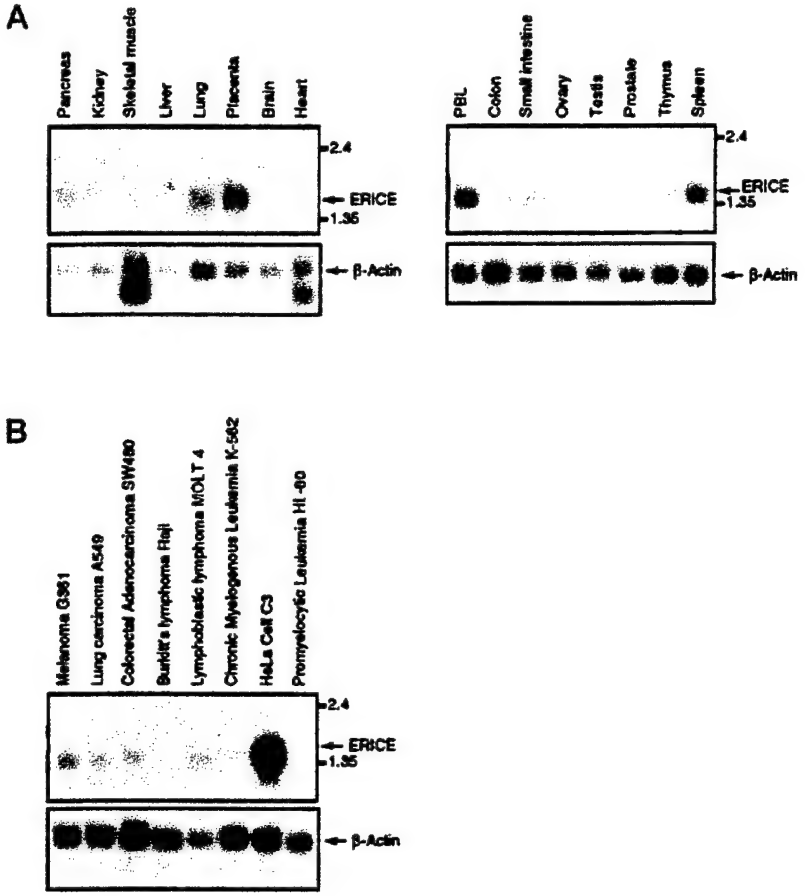
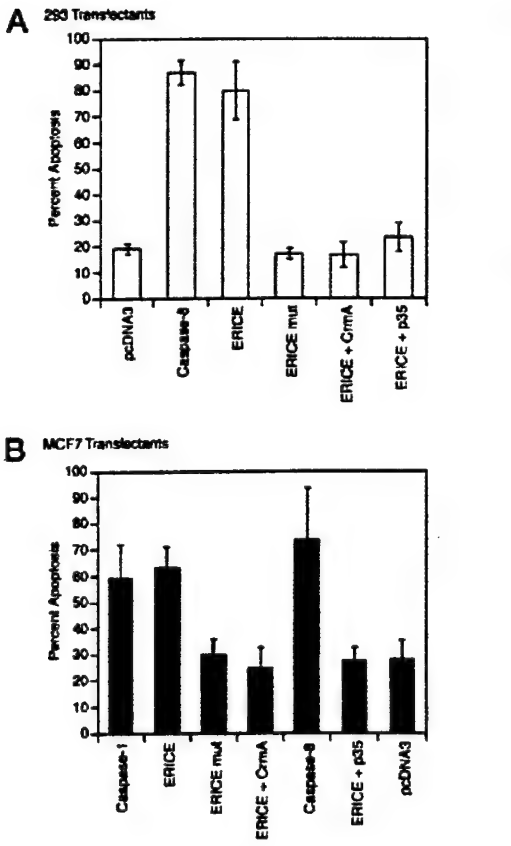


FIG. 2. **Tissue distribution of ERICE.** Human adult multiple tissue (*panel A*) and human multiple cancer cell line (*panel B*) poly(A)$^+$ Northern blots (CLONTECH) were probed with ^{32}P-labeled ERICE cDNA. The *upper arrow* represents the 1.6-kilobase ERICE mRNA transcript. The 1.35- and 2.4-kilobase size markers for mRNA are shown.



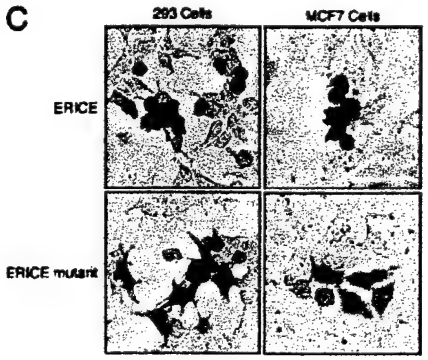


FIG. 3. **Overexpression of ERICE induces cell death in mammalian cells.** *Panel A*, 293 embryonic kidney cells were transiently transfected with the reporter gene β-galactosidase and wild type ERICE or an active site mutant of ERICE. Native ERICE was also overexpressed with virally encoded caspase inhibitors CrmA or p35. Percent apoptosis represents the mean value from three independent experiments (mean ± S.D.). *Panel B*, MCF7 breast cell carcinoma cells were processed as above. *Panel C*, transfected cells were stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside and examined by phase-contrast microscopy.

apoptosis in either the MCF7 or 293 cells. Furthermore, apoptosis induced by ERICE was efficiently blocked by virally encoded caspase inhibitors p35 and CrmA (Fig. 3, *A* and *B*).

Activation of ERICE—Although overexpressed ERICE induces apoptosis, this may not reflect its primary function because other ICE subfamily members do not appear to play a

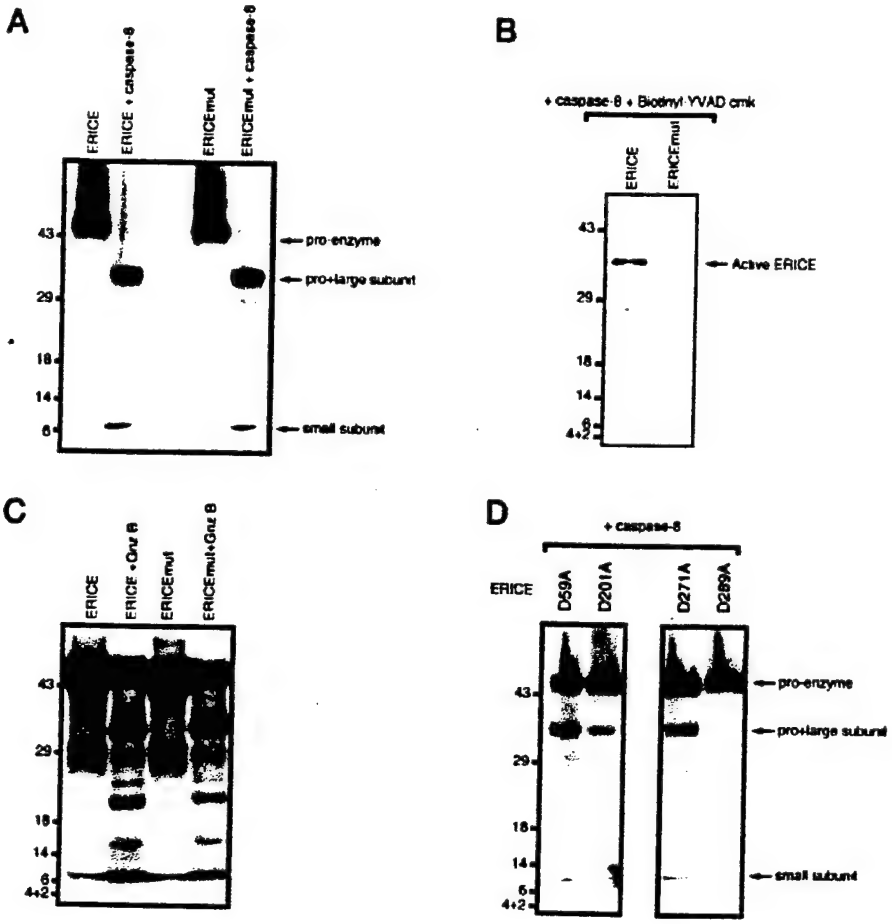


FIG. 4. **Activation of ERICE.** *Panel A*, ERICE and catalytically inactive ERICE cysteine mutant (C258S) are substrates for caspase-8 *in vitro*. *In vitro* translated ERICE or ERICE mutant was incubated in cleavage buffer and caspase-8 as detailed under "Materials and Methods." *Panel B*, activation of ERICE by caspase-8 cleavage. Caspase-8-cleaved products were incubated with biotinylated YVAD cholormethyl ketone and then immunoprecipitated using streptavidin beads as outlined under "Materials and Methods." Only the caspase-8-cleaved products of native ERICE were labeled with biotin-YVAD. *Panel C*, ERICE and ERICE cysteine mutant are proteolyzed by granzyme B *in vitro*. *In vitro* translated ERICE or ERICE mutant was incubated in cleavage buffer and granzyme B as detailed under "Materials and Methods." *Panel D*, to determine further the difference in cleavage sites between the caspase-8-proteolyzed ERICE, putative aspartate residues were mutated to alanines and the ERICE mutants reincubated with caspase-8.

prominent proapoptotic role. In keeping with this, granzyme B, which is known to induce apoptosis through the productive processing of caspases, cleaves but does not activate caspase-1, the prototypical member of the ICE subfamily. Additionally, members of the ICE subfamily are not productively processed by caspase-8, the apical caspase involved in proximal death receptor signaling. To address whether ERICE was productively processed by granzyme B or caspase-8, it was incubated with either protease (Fig. 4, *A* and *C*), and the emergence of active ERICE was assessed by reaction with biotinylated YVAD chloromethyl ketone, which covalently binds the catalytic cysteine within the large subunit of proteolytically competent (active) caspases (Fig. 4*B*). Caspase-8 processing of ERICE led to the generation of two subunits. One of the subunits was the prodomain plus the large catalytic subunit (pro+large), and the other was the small catalytic subunit. This is similar to the activation of caspase-1 in which the p45 zymogen must initially be processed to a stable active p35 pro+large subunit. Further processing, namely cleavage between the pro and large subunit, is highly dilutional sensitive and very inefficient *in vitro* such that *in vitro* translated zymogens do not undergo complete processing. Given the low concentration of *in vitro* translated ERICE, it was not surprising that caspase-8 processed it only to the pro+large and small subunits. Regardless, caspase-8-processed ERICE was efficiently labeled with biotinylated YVAD, indicative of generation of active ERICE (Fig. 4*B*). Granzyme B appeared to be more promiscuous, cleaving ERICE into a number of discrete fragments (Fig. 4*C*), none of which labeled with biotinylated YVAD (data not shown). *In vivo*, mutant ERICE was processed by both caspase-8 and Fas in a CrmA-inhibitable fashion (Fig. 5). Therefore, although ERICE is cleaved by both caspase-8 and granzyme B, it is only cleavage by caspase-8 which is productive and leads to the generation of active ERICE.

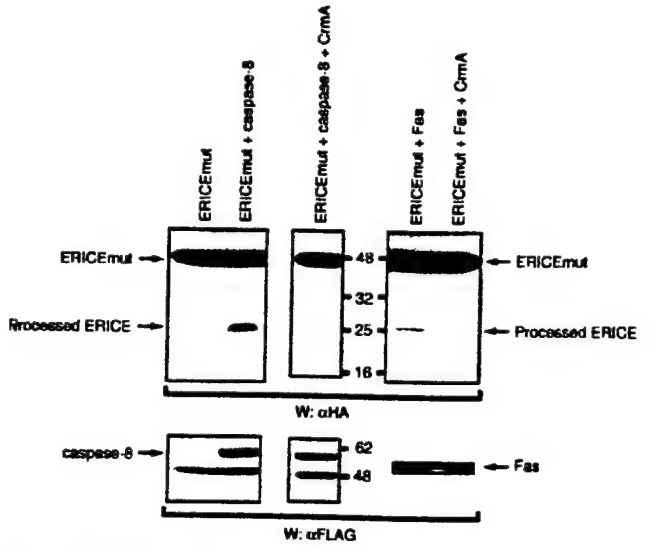


FIG. 5. ***In vivo* processing of ERICE is CrmA-inhibitable.** Catalytically inactive ERICE was cotransfected with caspase-8 or Fas in the presence or absence of CrmA. The formation of subunits was monitored by Western blotting for the hemagglutinin epitope-tagged ERICE. The expression of caspase-8 and Fas is shown in the *lower panels*.

To map caspase-8 processing sites within ERICE, potential aspartate cleavage sites were mutated and tested as caspase-8 substrates. Productive cleavage was found to require Asp^{289} as alteration of this residue abolished processing (Fig. 4*D*). Therefore, ERICE must be cleaved by caspase-8 following Asp^{289} to yield an active heterodimeric enzyme. Notably, this aspartate is found in the sequence context LEED (residues 286–289) which is the preferred substrate for caspase-8 cleavage. In summary, this is the first example of an ICE subfamily member

that is activated by caspase-8 and suggests a potential downstream role for active ERICE in caspase-8-mediated cell death.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 273, No. 5, Issue of January 30, pp. 2926–2930, 1998
Printed in U.S.A.

An Induced Proximity Model for Caspase-8 Activation*

(Received for publication, October 30, 1997)

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The assembly of the CD-95 (Fas/Apo-1) receptor death-inducing signaling complex occurs in a hierarchical manner; the death domain of CD-95 binds to the corresponding domain in the adapter molecule Fas-associated death domain (FADD) Mort-1, which in turn recruits the zymogen form of the death protease caspase-8 (FLICE/Mach-1) by a homophilic interaction involving the death effector domains. Immediately after recruitment, the single polypeptide FLICE zymogen is proteolytically processed to the active dimeric species composed of large and small catalytic subunits. Since all caspases cleave their substrates after Asp residues and are themselves processed from the single-chain zymogen to the two-chain active enzyme by cleavage at internal Asp residues, it follows that an upstream caspase can process a downstream zymogen. However, since FLICE represents the most apical caspase in the Fas pathway, its mode of activation has been enigmatic. We hypothesized that the FLICE zymogen possesses intrinsic enzymatic activity such that when approximated, it autoprocesses to the active protease. Support for this was provided by (i) the synthesis of chimeric F_{pk}3FLICE molecules that can be oligomerized *in vivo* by the synthetic cell-permeable dimerizer FK1012H2. Cells transfected with F_{pk}3FLICE underwent apoptosis after exposure to FK1012H2; (ii) the creation of a nonprocessable zymogen form of FLICE that retained low but detectable protease activity.

Apoptosis, or programmed cell death, is a cell deletion mechanism that is critical to metazoan survival (1, 2). The cell death machinery is conserved throughout evolution and is composed of several distinct parts including effectors, inhibitors, and activators (1, 3).

Mammalian cysteine proteases (designated caspase for cysteine aspartic acid-specific protease) related to the *Caenorhabditis elegans* cell death gene *CED-3* represent the effector components of the apoptotic machinery participating in a regulated proteolytic cascade (4, 5). Since all caspases cleave their substrates after Asp residues and are themselves processed from the single-chain zymogen to the two-chain active enzyme by cleavage at internal Asp residues, it follows that an upstream caspase can process a downstream zymogen (6–8).

The assembly of the Fas receptor death-inducing signaling complex occurs in a hierarchical manner; the death domain of CD-95 binds to the corresponding domain in the adapter molecule Fas-associated death domain (FADD) Mort-1, which in turn recruits the zymogen form of the death protease caspase-8 (FLICE/Mach-1) by a homophilic interaction involving the death effector domains (9–12). Immediately after recruitment, the single polypeptide FLICE zymogen is proteolytically processed to the active dimeric species composed of large and small catalytic subunits that amplify the apoptotic signal by activating other downstream caspases (6, 8, 13). However, since FLICE represents the most apical caspase in the Fas pathway, its mode of activation has been enigmatic.

We hypothesized that the FLICE zymogen possesses intrinsic enzymatic activity such that when approximated, it autoprocesses to the active protease. Support for this model was provided by two independent approaches: (i) the synthesis of chimeric F_{pk}3FLICE molecules that can be oligomerized *in vivo* by the synthetic cell-permeable dimerizer FK1012H2. Cells transfected with F_{pk}3FLICE underwent apoptosis after exposure to FK1012H2; (ii) the creation of a nonprocessable zymogen form of FLICE that retained low but detectable protease activity.

MATERIALS AND METHODS

Expression Vectors and Recombinant Proteins—$MF_{pk}3E_c$ vector containing a myristoylation site, three copies of F_{pk} in tandem and a hemagglutinin epitope tag (HA) was originally made by D. Spencer (Baylor College of Medicine). F_{pk} is a double mutant of FKBP12 where residues 89 and 90 have been mutated from GI to PK (14). The catalytic domain of FLICE (encoding Ser-217 to Asp-479) was obtained by polymerase chain reaction and sub-cloned in-frame between the last F_{pk} and the epitope tag at the *Sal*I site in $MF_{pk}3E_c$. The same catalytic domain of a mutant version of FLICE, in which Cys-360 was replaced by Ser, was similarly cloned. For production of recombinant purified proteins, the catalytic domain of FLICE or mutant versions of FLICE in which the catalytic Cys-360 was replaced by Ser and/or the cleavage sites Asp-374 and Asp-384 were replaced by Ala, were obtained by polymerase chain reaction and sub-cloned into the pET15b expression vector (Novagen). The proteins were expressed in the BL21 pLysS *Escherichia coli* strain and purified using the QIAexpress Kit (Qiagen) following the manufacturer's instructions.

Cells and Transfections—Human embryonic kidney 293 and HeLa cells were cultured as described previously. Cell death assays were performed as described (9). Hela cells were transfected using the lipofectAMINE procedure (Life Technologies, Inc.) according to the manufacturer's instructions. 293 cells were transfected using calcium phosphate precipitation.

Western and Coimmunoprecipitation Analysis—For immunoblotting analysis, cells (5×10^5) were lysed in 50 μl of buffer (50 mM KCl, 50 mM HEPES, 5 mM EGTA, 2 mM $MgCl_2$, protease inhibitor mixture) followed by three cycles of freeze thaw. The membrane-rich fraction was pelleted, resuspended in 8 M urea and, after boiling in sample buffer, resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and probed with anti-HA antibody. For coim-

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§ Supported by a Human Frontier Science Program Organization (HFSPO) fellowship.

‖ A Howard Hughes Medical Institute predoctoral fellow supported in part by NIGMS, National Institutes of Health, Grant GM-52067 (to S. L. Schreiber).

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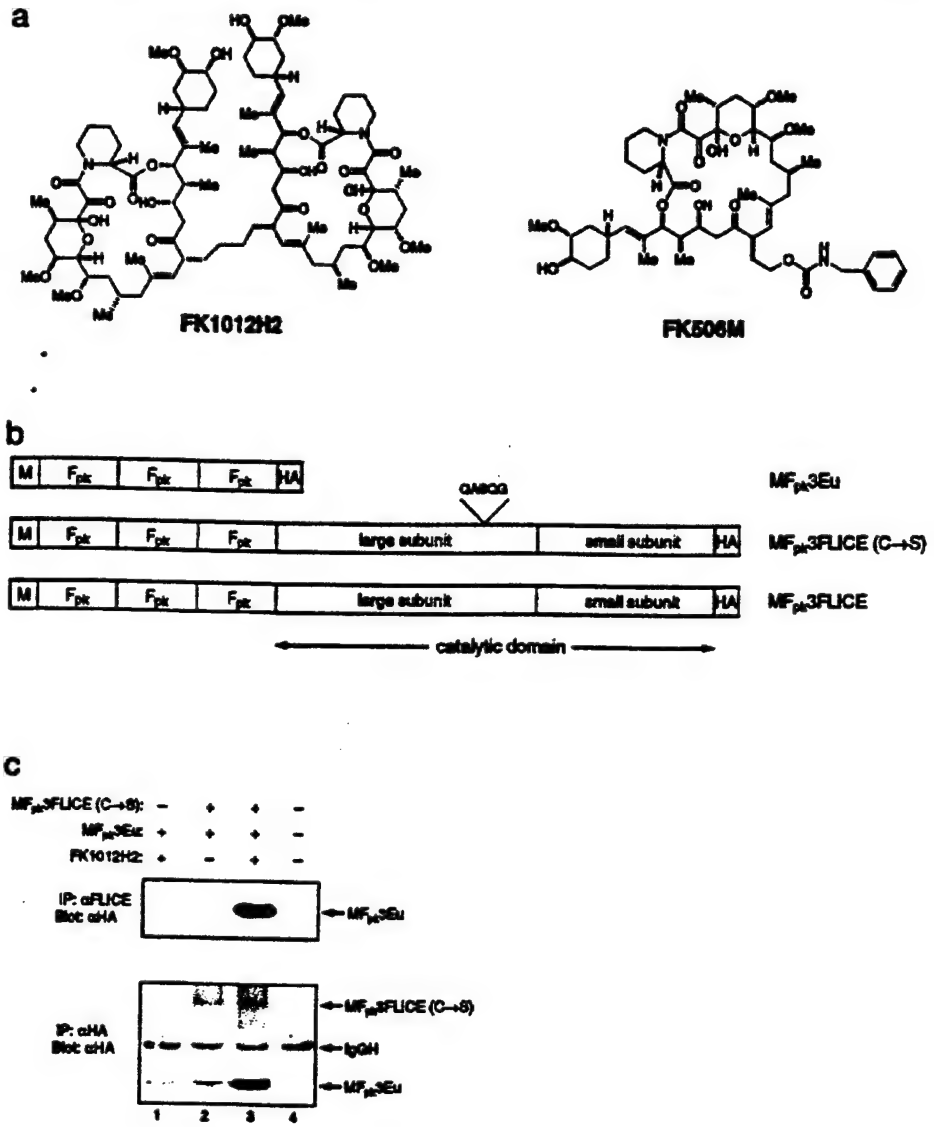

FIG. 1. **Induced oligomerization of F_{pk} containing proteins.** *a*, structure of the synthetic ligands; FK1012H2 is a dimeric variant of FK506M. *b*, schematic representation of F_{pk}-containing chimeric proteins; *M* indicates a myristoylation site; HA, hemagglutinin epitope tag; $F_{pk}3$, three mutant FKBP polypeptides in tandem. $MF_{pk}3FLICE$ contains three F_{pk} polypeptides fused to the catalytic domain of FLICE. $MF_{pk}3FLICE(C \rightarrow S)$ is a mutant version of $MF_{pk}3FLICE$ where the catalytic Cys-360 has been replaced by Ser (9). *c*, FK1012H2-induced oligomerization of F_{pk}-containing proteins. 293 cells were transiently transfected with $MF_{pk}3E_u$ alone (*lane 1*), $MF_{pk}3E_u$ and $MF_{pk}3FLICE(C \rightarrow S)$ (*lanes 2* and *3*), or vector alone (*lane 4*). After treatment with FK1012H2 (*lanes 1* and *3*), cells were lysed and anti-FLICE (*upper panel*) or anti-HA (*lower panel*) immunoprecipitates (*IP*) were immunoblotted with anti-HA monoclonal antibody. In the absence of FK1012H2 (*lane 2*), no complex is detectable between $MF_{pk}3FLICE(C \rightarrow S)$ and $MF_{pk}3E_u$. In the presence of the dimerizer FK1012H2 (*lane 3*), complex formation is evident.

munoprecipitation analysis, cells (2×10^6) were lysed in 1 ml of buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris, 20 mM HEPES, protease inhibitor mixture) and incubated either with anti-HA[1] antibodies or anti-FLICE (small subunit-specific) rabbit antiserum. Immune complexes were precipitated by the addition of protein G-Sepharose (Sigma). After extensive washing, the Sepharose beads were boiled in sample buffer, and the eluted proteins were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-HA antibody.

Enzymatic Analysis of Recombinant Mutant Proteins—The enzymatic reaction was carried out at 37 °C in 20 mM PIPES, 0.1 mM substrate, 100 mM NaCl, 10 mM dithiothreitol (fresh), 1 mM EDTA, 0.1% CHAPS, and 10% sucrose, pH 7.2. The initial rates of hydrolysis were measured by release of AFC (7-amino-4-methyl cou marin) from the substrate by the enzymes at appropriate emission and excitation wavelengths using a Perkin-Elmer LS50B fluorimeter equipped with a thermostated plate reader. Titrations of wild type and FLICE-DD [arrow] AA were carried out by preincubating the enzyme with varying concentrations of Z-DEVD-FMK or CrmA in assay buffer for 30 min at room temperature. The inhibitor was added at concentrations spanning from 0 to significantly above the concentration of active enzyme. After the preincubation, Z-DEVD-AFC was added in assay buffer to a final substrate concentration of 0.1 mM. The relative amount of uninhibited enzyme was evaluated from the initial rates of hydrolysis of the substrate as described above, and the concentration of active enzyme was calculated by extrapolating data points to their intersection with the *x* axis. The caspase-8 concentration used in the titration assays were 0.2 micromolar for the native enzyme and 20 micromolar for the DD → AA mutant.

Affinity Labeling of Recombinant Mutant Proteins—100 ng of purified proteins were incubated with or without N-(biotinyl-Asp-Glu-Val-Asp-[(2,6-dimethylbenzoyl)oxy]methyl ketone (BIO-DEVD-AMK) (15) at a concentration of 0.5 μM in a final volume of 50 μl of buffer (0.1% CHAPS, 10 mM dithiothreitol, 10 mM Tris, pH 7.5) for 15 min at 25 °C. For competition experiments, increasing amounts of a nonbiotinylated version of the DEVD tetrapeptide (DEVD-CMK; Bachem) were added to the reaction. Samples were boiled in sample buffer in the absence of reductant, resolved by SDS-polyacrylamide gel electrophoresis under nonreducing conditions, blotted onto nitrocellulose, blocked in phosphate-buffered saline containing 3% bovine serum albumin and 0.1% Tween 20, incubated with streptavidin-conjugated horseradish peroxidase (ICN) in phosphate-buffered saline, 0.1% bovine serum albumin, 0.1% Tween, washed with 50 mM Tris, pH 7.5, 0.25% gelatin, 0.05% Tween 20, 150 mM NaCl, 5 mM EDTA, and developed by ECL. Alternatively, the membrane was analyzed by immunoblotting with anti-FLICE antiserum specific for the small catalytic subunit.

RESULTS AND DISCUSSION

To determine if FLICE oligomerization *in vivo* could result in activation, chimeric F_{pk}FLICE expression constructs were engineered. F_{pk} (molecular mass 12 kDa), a double mutant of FKBP (FK binding protein FK506) (16) contains a single binding site for the cell-permeable immunosuppressive drug FK506. The FKBP-FK506 complex is a potent inhibitor of calcineurin, a protein phosphatase that plays a key role in signal-

[1] The abbreviations used are: HA, hemagglutinin; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; BIO-DEVD-AMK, N-(biotinyl-Asp-Glu-Val-Asp-[(2,6-dimethylbenzoyl)oxy]methyl ketone; -CMK, chloromethyl ketone; -FMK, fluoromethyl ketone; -AFC, 7-amino-4-trifluoromethyl coumarin; Z-, carbobenoxy.

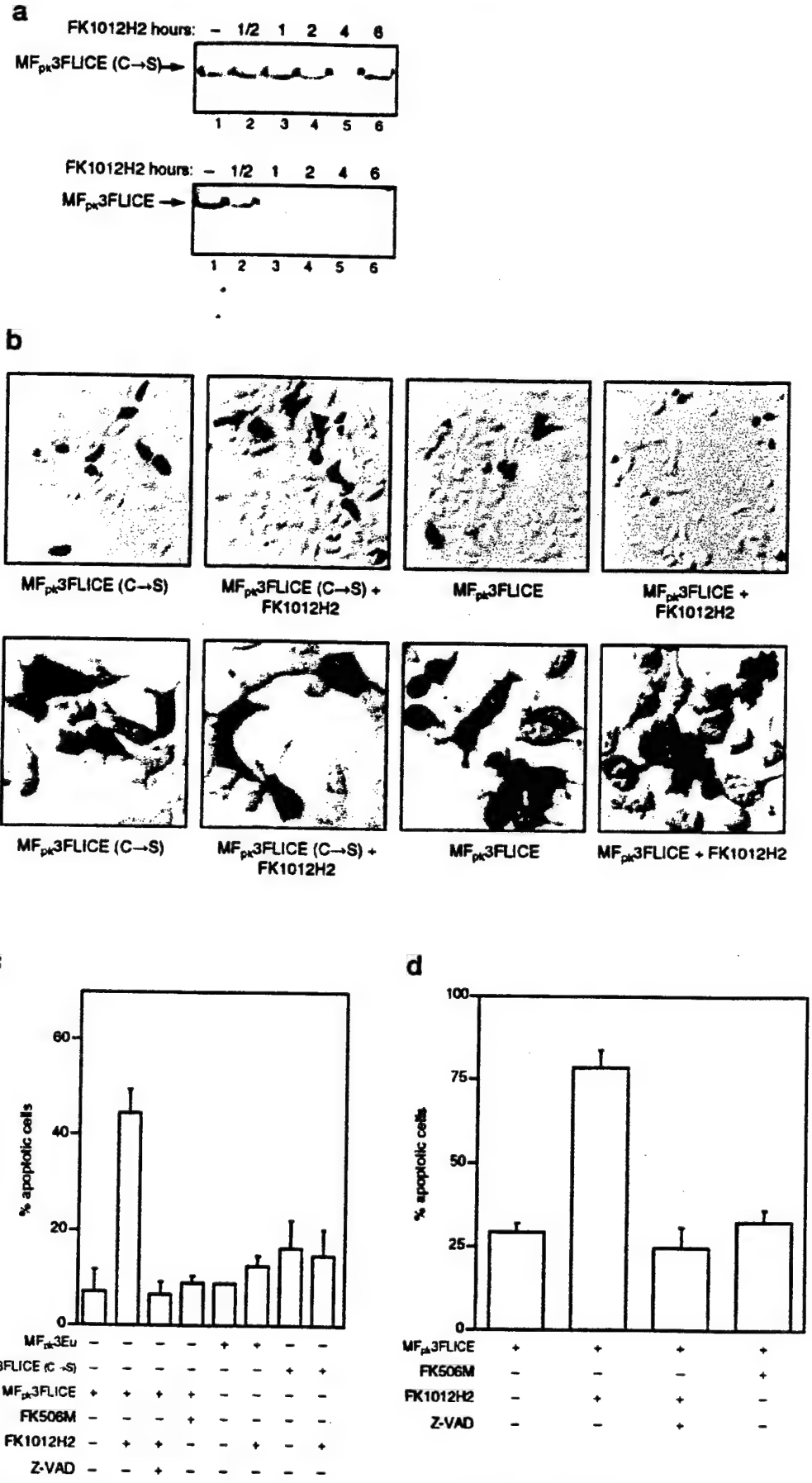

FIG. 2. **FK1012H2-induced oligomerization of FLICE triggers self-processing and apoptosis.** *a*, 293 cells were transiently transfected with MF$_{pk}$3FLICE(C → S) (*upper panel*) or MF$_{pk}$3FLICE (*lower panel*); 36 h post-transfection cells were treated with FK1012H2 (250 nM) for the indicated times. Cell extracts equalized for protein content were analyzed by immunoblotting with anti-HA monoclonal antibody. *b*, 293 and HeLa cells were transiently transfected with MF$_{pk}$3FLICE(C → S) or MF$_{pk}$3FLICE with pCMV-β-galactosidase. 36 h post-transfection cells were left untreated or treated with FK1012H2 (250 nM) for 3 h, fixed, stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal), and

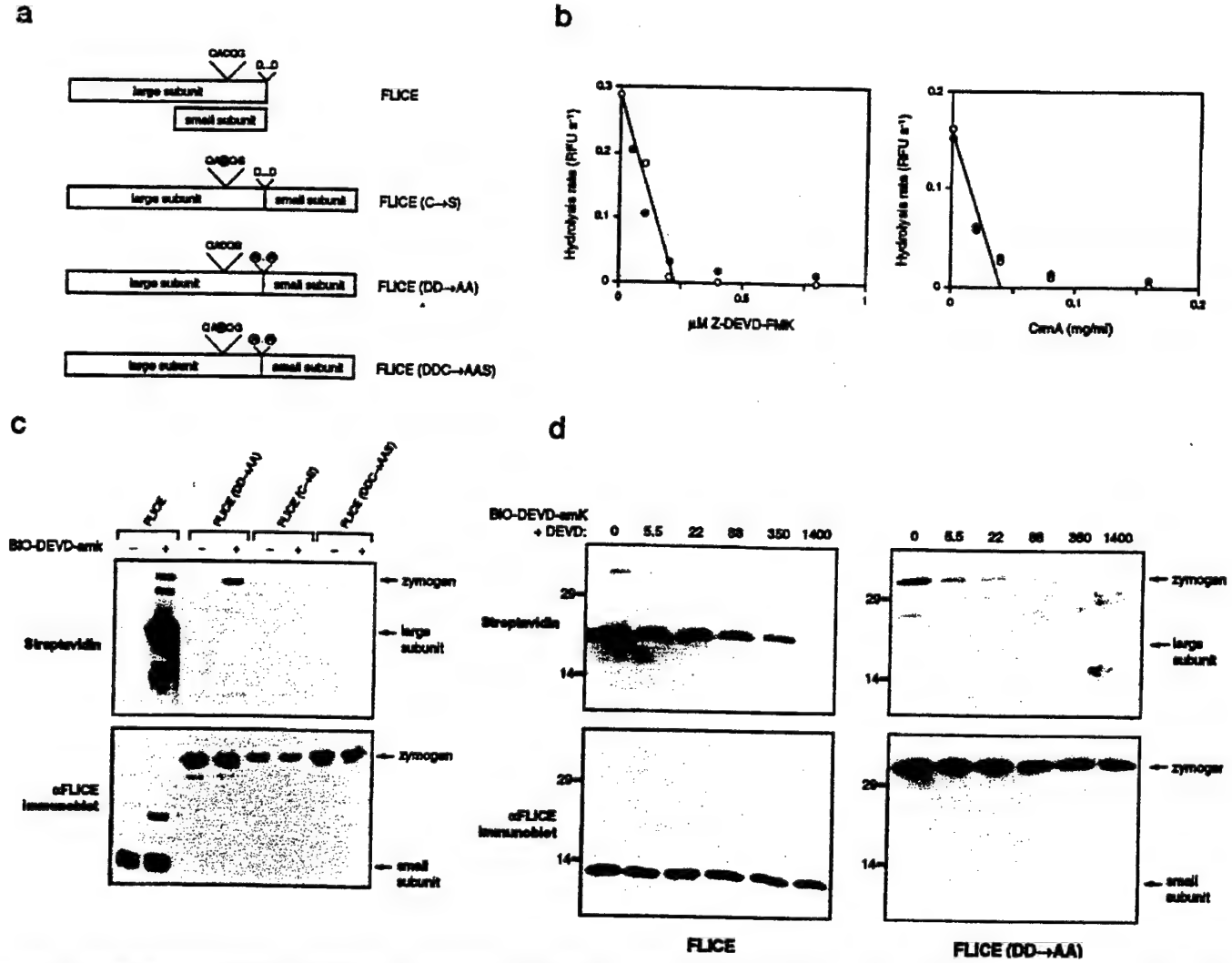

FIG. 3. **Titration and affinity labeling of recombinant FLICE.** *a*, schematic representation of the different mutant versions of FLICE; mutated residues are *circled*. *b*, FLICE(DD → AA) enzyme was titrated with the inhibitors Z-DEVD-FMK (*left panel*) and CrmA (*right panel*). Equivalent amounts of activity were added for the wild type (*open circles*) and mutant enzymes (*solid circles*), corresponding to a 100-fold higher protein concentration for the mutant enzyme. *c*, recombinant purified native active FLICE composed predominantly of the large and small catalytic subunits, and indicated mutated zymogen forms were compared for their ability to bind the biotinylated irreversible tetrapeptide inhibitor DEVD-AMK (BIO-DEVD-AMK) as detected by Western blot analysis using horseradish peroxidase HRP-streptavidin (*upper panel*). Immunoblot analysis with an anti-FLICE (small catalytic subunit-specific) antiserum of the same samples is shown as a control (*lower panel*). As expected, the large catalytic subunit of processed native FLICE that contains the active-site cysteine avidly bound the biotinylated inhibitor. The nonprocessable zymogen form of FLICE(DD → AA) that also contains the active-site cysteine bound the inhibitor, albeit to a lesser extent, consistent with a model in which the zymogen possesses enzymatic activity. As predicted, mutant forms lacking the catalytic cysteine (C → S and DDC → AAS) did not bind the inhibitor. *d*, specificity of the interaction between BIO-DEVD-AMK and FLICE or FLICE(DD-AA) was confirmed by competing the binding with increasing amounts of a nonbiotinylated version of the DEVD tetrapeptide (5.5–1,400-fold excess of DEVD with respect to BIO-DEVD). Samples were analyzed as in *c*.

ing. An FK506 dimer (FK1012H2) was previously synthesized by introducing a cross-linker into the domain of FK506 necessary for inhibition of calcineurin. FK1012H2, although unable to bind calcineurin, still retains its ability to bind and dimerize F_{pk} polypeptides given its bivalent nature (17–19) (Fig. 1*a*).

To mimic recruitment of FLICE to its receptor signaling complex (11, 12), the prodomain of FLICE was substituted with a myristoylation signal followed by three tandem repeats of F_{pk}, ($MF_{pk}3$FLICE). A catalytically inactive version was constructed by mutating the active-site Cys-360 to Ser ($MF_{pk}3$FLICE(C → S)). An additional control was the construct $MF_{pk}3E_u$ that encoded only the myristoylation signal, three F_{pk} repeats in tandem, and an HA epitope tag (Fig. 1*b*). To confirm the ability of FK1012H2 to induce oligomerization of $F_{pk}3$-containing proteins, human 293 cells were transiently transfected with the $MF_{pk}3E_u$ construct and the catalytically inactive chimera $MF_{pk}3$FLICE(C → S). The $MF_{pk}3$FLICE immunoprecipitates contained associating $MF_{pk}3E_u$ only in the presence of FK1012H2 (Fig. 1*c*), confirming the validity of the dimerization approach.

Ectopic expression of the catalytically active chimera $MF_{pk}3$FLICE resulted in the production of a protein of predicted molecular mass (69 kDa) that was membrane-associated. The addition of the synthetic ligand FK1012H2 induced

photographed under phase contrast microscopy. *c*, 293 cells were transiently transfected with $MF_{pk}3E_u$, $MF_{pk}3$FLICE, or $MF_{pk}3$FLICE(C → S) and pCMV-β-galactosidase. 36 h post transfection cells were treated with FK1012H2 (250 nM) or FK506 M (250 nM) with or without the addition of the broad-spectrum caspase inhibitor Z-VAD-FMK (20 μM). After 3 h, cells were fixed, stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) and examined by phase contrast microscopy. Data (mean ± S.E.) shown are the percentage of round and blebbing apoptotic cells as a function of total number of blue cells counted ($n > 3$). *d*, HeLa cells were treated and analyzed as in *c*.

rapid disappearance of the catalytically competent MF_{pk}3FLICE chimera. Emergence of the active subunits, however, was not detectable under these experimental conditions. As predicted, the catalytically inactive derivative was efficiently expressed, but on exposure to FK1012H2, did not undergo processing even after prolonged incubation (Fig. 2*a*).

We next observed if MF_{pk}3FLICE oligomerization resulted in the expected apoptotic demise of transfected cells. Human 293 and HeLa cells were transiently transfected with MF_{pk}3FLICE-(C → S) or MF_{pk}3FLICE expression constructs together with a reporter plasmid encoding β-galactosidase. Thirty six h after transfection, cells were left untreated or treated with 250 nM FK1012H2, stained, and analyzed by phase contrast microscopy. As shown in Fig. 2*b*, oligomerization of MF_{pk}3FLICE but not MF_{pk}3FLICE(C → S) induced phenotypic alterations characteristic of apoptosis. Cells shrank, displayed membrane blebbing, and detached from the dish. Additionally, the apoptotic substrate poly(ADP-ribose) polymerase was cleaved to its signature 85-kDa form, and genomic DNA was cleaved into characteristic internucleosomal size fragments (data not shown), both biochemical hallmarks of apoptosis. Significantly, treatment of Fpk or Fpk-FLICE(C → S)-transfected cells with higher doses of FK1012H2 for an extended period of time did not induce apoptosis (16, 17, 19, 20) (data not shown). After 3 h of exposure to the synthetic ligand, 50–80% MF_{pk}3FLICE-transfected cells started blebbing, condensing, and detaching from the dish. This was completely abrogated by the broad-spectrum caspase inhibitor Z-VAD-FMK that has previously been shown to inhibit FLICE-induced apoptosis (Fig. 2, *c* and *d*) (12). Importantly, the monomeric form of the ligand, FK506M, did not induce apoptosis, confirming that the results observed were oligomerization-dependent (Fig. 2, *c* and *d*).

These data demonstrate that specific clustering of FLICE zymogen causes apoptosis through caspase activation. We hypothesized that this activation occurs through self-processing of the clustered FLICE due to an intrinsic proteolytic activity of the zymogen. To further address this hypothesis, different recombinant versions of FLICE were generated (Fig. 3*a*); as shown previously (6, 8, 11), overexpression of catalytically competent FLICE constructs in *E. coli* generated an active heterodimeric enzyme composed of large and small catalytic subunits. Enzymatically inactive FLICE (catalytic Cys-360 mutated to Ser; FLICE(C → S)) did not undergo processing, consistent with a requirement for intrinsic enzymatic activity. Importantly, an altered form of FLICE that retained the catalytic cysteine but was mutated at the internal cleavage sites (Asp-374 and Asp-384; FLICE(DD → AA)) also did not undergo auto-processing and was expressed as a single polypeptide zymogen as confirmed by protein staining and anti-FLICE immunoblot analysis (data not shown and Fig. 3*c*). The processing mutant FLICE in which the catalytic cysteine was additionally inactivated (FLICE (DDCAAS)) was also expressed as a single polypeptide zymogen. These recombinant forms of FLICE were used to establish whether the zymogen did indeed possess enzymatic activity.

The activity of the cleavage site mutant version FLICE(DD → AA) was determined by its ability to hydrolyze the tetrapeptide caspase substrate Z-DEVD-AFC (21), and the portion of active material was determined by titration with the protein inhibitor CrmA (22, 23) and the covalent peptide based inhibitor, Z-DEVD-FMK (21). A 100-fold more FLICE(DD → AA) was required on a protein basis to give the same rates of substrate hydrolysis as wild type FLICE (Fig. 3*b*). Thus, from these results it is apparent that the bacterially expressed FLICE(DD → AA) mutant equivalent to the zymogen form has approximately 1% of the activity of the wild type enzyme. As expected, the catalytic mutant FLICE(C → S) possessed no enzymatic activity (data not shown).

To interpret the enzymatic analysis, we tested the ability of the different versions of FLICE to bind the biotinylated irreversible inhibitor BIO-DEVD-AMK, which covalently binds to the active-site cysteine (15). As expected, the large subunit of processed native FLICE that contains the catalytic cysteine bound BIO-DEVD-AMK (Fig. 3*c*). Additionally, the unprocessed cleavage site mutant (FLICE(DD → AA)) also bound BIO-DEVD-AMK, indicating that the 1% activity resides in the single chain. FLICE(DDC → AAS), as anticipated, did not show any specific binding to BIO-DEVD-AMK. The specificity of the bands indicated was confirmed by competing the binding with a nonbiotinylated version of the DEVD tetrapeptide (Fig. 3*d*).

Taken together, these data are consistent with a model wherein FLICE zymogen possesses intrinsic low level caspase activity that upon approximation mediated by the adapter molecule Fas-associated death domain (FADD) attains a sufficient concentration to activate the apoptosis pathway. This study provides a remarkably simple solution to the chicken and egg conundrum of how the initiating caspase (FLICE) is proteolytically processed.

Acknowledgments— We are grateful to S. L. Schreiber for helpful suggestions and discussions. We thank David Spencer for $MF_{pk}3E_u$, Linda Clayton for BIO-DEVD, Ian Jones for his expertise in preparing the figures, and the following members of the Dixit lab for encouragement and discussions: Divya Chaudhary, Arul Chinnaiyan, Hangjun Duan, Shimin Hu, Eric Humke, Justin McCarthy, Karen O'Rourke, James Pan, and Claudius Vincenz.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 10, Issue of March 6, pp. 5841–5845, 1998
Printed in U.S.A.
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Caspase-9, Bcl-X_L, and Apaf-1 Form a Ternary Complex*

(Received for publication, November 18, 1997, and in revised form, December 24, 1997)

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Genetic analysis of apoptosis in the nematode *Caenorhabditis elegans* has revealed the cell death machine to be composed of three core interacting components. CED-4 (equivalent to mammalian Apaf-1) is a nucleotide binding molecule that complexes with the zymogen form of the death protease CED-3, leading to its autoactivation and cell death. CED-9 blocks death by complexing with CED-4 and attenuating its ability to promote CED-3 activation. An equivalent ternary complex was found to be present in mammalian cells involving Apaf-1, the mammalian death protease caspase-9, and Bcl-X_L, an anti-apoptotic member of the Bcl-2 family. Consistent with a central role for caspase-9, a dominant negative form effectively inhibited cell death initiated by a wide variety of inducers.

Programmed cell death, or apoptosis, is an evolutionarily conserved and genetically regulated biological process that plays an important role in the development and homeostasis of multicellular organisms (1–4). The nematode *Caenorhabditis elegans* has served as a model system for defining core components of the death machine (5, 6). CED-3 represents the effector arm of the cell death machine and belongs to a family of related mammalian proteases termed caspases for cysteine proteases that cleave following an Asp residue (7, 8). Caspases exist as zymogens composed of a prodomain plus large and small catalytic subunits. Generation of the active enzyme requires accurate processing at internal Asp residues to liberate the prodomain and produce the two chain active enzyme (7–9). Caspases can be classified according to whether they possess a large or a small prodomain. Large prodomains function as signal integrators as they bind adapter molecules involved in signal transduction. For example, the death effector domain within the prodomain of caspase-8 binds to the corresponding motif in the adapter molecule FADD[1] allowing for its recruitment to the CD-95 death receptor signaling complex (10, 11).

The death effector domain is a specific example of a more global homophilic interaction domain termed CARD (for caspase recruitment domain) that is present in other large prodomains including those of caspase-2 (ICH-1) and caspase-9 (ICE-LAP6, Mch6; Ref. 12). Caspase-2 is recruited to the TNFR-1 signaling complex through an interaction involving the respective CARD domains within the adapter molecule RAIDD and the prodomain of caspase-2 (13). To date, the other large prodomain-containing caspase, caspase-9, has not been implicated in any specific signaling pathway (14). We find that caspase-9 is part of a ternary signaling complex analogous to the one present in *C. elegans* involving CED-3, CED-4, and CED-9 (6, 15–17). CED-9 is an inhibitor of apoptosis in the nematode and corresponds to mammalian cell death inhibitors including Bcl-2 and Bcl-X_L (18). It can be found complexed with the nematode caspase equivalent CED-3 in the presence of the bridging molecule CED-4 (15–17). This suggests that a molecular mechanism based on the physical interaction of these components could potentially account for the inhibitory function of CED-9 and, by extension, Bcl-X_L and Bcl-2 (5). The ability of the worm genes to function in mammalian cells underscores their conservation and the interchangeability of key death components (15, 16). For example, in transfected human embryonic kidney cells, CED-4 bound CED-9 or its mammalian counterpart Bcl-X_L. Similarly, CED-4 bound CED-3 or corresponding large prodomain mammalian caspases (including caspase-1 and caspase-8) but not small prodomain caspases like caspase-3. The inability of dominant negative versions of caspase-1 or caspase-8 to block CED-4-induced cell death suggested that either another distinct large prodomain caspase was the primary target or that CED-4 activated multiple caspases (15). The exact mechanism deployed by CED-4 to activate CED-3 and/or caspases remains unclear. It has been shown, however, that CED-4 is a P-loop-containing nucleotide binding protein that is capable of promoting the activation of CED-3 and that this is blocked by CED-9 (19–21).

Recently, a human CED-4 homologue (Apaf-1) has been identified that possesses an NH_2-terminal CED-3 prodomain-like region that includes a CARD domain, a CED-4-like segment including conserved P-loop and a COOH-terminal extension composed of multiple WD-40 repeats that are lacking in nematode CED-4 (Fig. 1*A*; Ref. 22). Apaf-1, in the presence of cytochrome *c*, nucleotide (dATP), and a previously unidentified factor (Apaf-3) that has recently been shown to be caspase 9 (31), is able to promote the activation of caspase-3 (a small prodomain downstream caspase) by a mechanism that awaits definition (23–25). We found that caspase-9, but not other large prodomain caspases, and Bcl-X_L bound distinct regions in Apaf-1 and that dominant negative caspase-9 effectively blocked cell death induced by a variety of effectors. Thus, caspase-9 likely represents a direct downstream target of Apaf-1 and its activation appears critical for the propagation of death signals.

EXPERIMENTAL PROCEDURES

Expression Constructs—cDNAs encoding Apaf-1 or its truncated forms were obtained by polymerase chain reaction based on the published Apaf-1 DNA sequence (22). The full-length Apaf-1 was cloned into pcDNA3 (Invitrogen) with a NH_2-terminal Flag tag. Apaf-1(3+4)-Myc (amino acids 1–412), Apaf-1(3)-Myc (amino acids 1–102), and Apaf-1(4)-Myc (amino acids 86–412) were cloned into pcDNA3.1(−)/Myc-His

* This work was supported by National Institutes of Health Grants ES08111 and DAMD 17-96-6085. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: FADD, Fas-associated death domain; RAIDD, RIP-associated ICH-1/CED-3-homologous protein with a death domain; TRADD, tumor necrosis factor-associated death domain. TNF, tumor necrosis factor; mAb, monoclonal antibody.

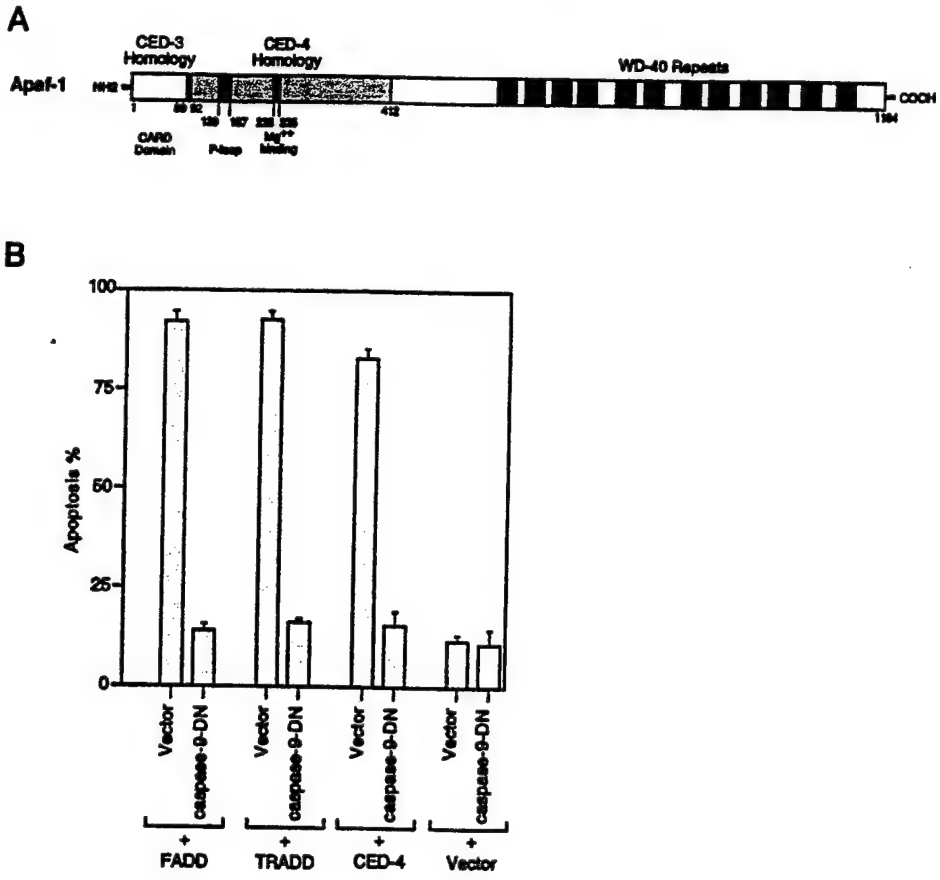

FIG. 1. **Dominant negative caspase-9 (*caspase-9-DN*) blocked apoptosis induced by FADD, TRADD, and CED-4.** *A*, a schematic presentation of Apaf-1. Apaf-1 contains an NH_2-terminal CED-3-homologous region (amino acids 1–89) that includes a CARD domain as indicated, a CED-4-homologous segment (amino acids 92–412) and a COOH-terminal sequence that includes 12 WD-40 repeats as shown. The P-loop sequence for nucleotide binding and a putative Mg^{2+} binding site are also indicated. *B*, FADD-, TRADD-, and CED-4-induced cell death is inhibited by caspase-9-DN. MCF7 cells were co-transfected with FADD, TRADD, or CED-4 together with a β-galactosidase-expressing reporter construct in the presence of a 3-fold excess of either vector or caspase-9-DN. Fifteen hours after transfection, cells were stained and examined as described (28). The data (mean ± S.D.) represent the percentage of round, apoptotic cells as a function of total β-galactosidase-positive cells ($n = 3$). *DN*, dominant negative.

B (Invitrogen) with a COOH-terminal Myc tag provided by the vector. The construct expressing caspase-9-prodomain (amino acids 1–168) was cloned by polymerase chain reaction into pcDNA3 with a COOH-terminal Flag tag. The constructs encoding HA-BAX, HA-BAK, HA-BIK, Bcl-XL-Myc, Bcl-X_L-Flag, Bcl-$X_L$mt1-Flag, Bcl-XLmt7-Flag, caspase-1-Flag, caspase-2-prodomain-Flag, caspase-3-Flag, caspase-8-DN-Flag, caspase-9-DN-Flag, caspase-9 p30-Flag (amino acids 130–416), CED-4-Myc, FADD, RAIDD, TRADD-Myc, cIAP1, and CrmA have been described elsewhere (11, 13–15, 26).

Apoptosis Assays—Cell death assays were performed as described (26, 27). MCF7 cells were transfected using the lipofectAMINE procedure (Life Technologies, Inc.) according to the manufacturer's instructions.

Co-immunoprecipitation—*In vivo* interaction assays have been described elsewhere (26, 28). 293 cells were transfected by means of calcium phosphate precipitation.

RESULTS AND DISCUSSION

Dominant Negative Caspase-9 (Caspase-9-DN) Blocks Apoptosis Induced by FADD, TRADD, and CED-4—As shown in Fig. 1*B*, dominant negative caspase-9 (caspase-9-DN) inhibited cell death induced by the receptor associated death adapter molecules FADD and TRADD in human breast carcinoma MCF7 cells, consistent with caspase-9 functioning downstream of these two adapter molecules. Importantly, CED-4-induced apoptosis, which had previously been shown not to be blocked by dominant negative forms of the other large prodomain caspases (including caspase-2 and caspase-8) (9), was blocked by dominant negative caspase-9. It is therefore probable that nematode CED-4 induces apoptosis in mammalian cells by activating caspase-9. In keeping with this observation, we have previously noted that caspase-9 physically interacts with CED-4 (14).

Caspase-9 but Not Other Caspases Interacts with Apaf-1—To determine if caspase-9 similarly bound the mammalian CED-4 equivalent Apaf-1, co-immunoprecipitation was undertaken in human embryonic kidney 293 cells. Flag-Apaf-1 was found to co-precipitate with caspase-9 (Fig. 2*A*). Since caspase-9-DN-Flag also bound truncated Apaf-1 (Apaf-1(3+4); residues 1–412) that contained only the CED-3 and CED-4 homologous regions (Fig. 2*B*, *left*), further analysis used only this truncated form. Caspase-9-DN-Flag, but not other large prodomain-containing caspases, specifically immunoprecipitated with Apaf-1 (Fig. 2*B*, *left*). Therefore, unlike CED-4, which appears to promiscuously bind large prodomain caspases, Apaf-1 was specific for caspase-9. Additionally, as expected, the small prodomain-containing caspase-3 did not bind Apaf-1. The specificity of this interaction was confirmed by the finding that caspase-2, a CARD-containing large prodomain caspase, bound its cognate adapter molecule RAIDD through a CARD-mediated interaction, yet did not interact with the Apaf-1 CARD domain (Fig. 2*B*, *right*). To delineate the Apaf-1 interacting domain in caspase-9, both a prodomainless form (caspase-9 p30-Flag) and a prodomain only expressing form were assessed in binding studies. Consistent with the expected involvement of the prodomain in recruitment to signaling complexes, only the prodomain of caspase-9 was required to interact with Apaf-1(3+4)-Myc (Fig. 2*C*).

Bcl-X_L Interacts with Apaf-1—Given that CED-9 binds CED-4 (15–17), we asked whether an equivalent interaction existed between the corresponding mammalian counterparts Bcl-X_L and Apaf-1. Upon co-transfection, Flag-Apaf-1 co-precipitated with Bcl-X_L-Myc (Fig. 2*A*). We consistently observed that Apaf-1 expression was enhanced by co-expressing Bcl-X_L (15), suggesting that Bcl-X_L may stabilize Apaf-1. Bcl-X_L-Flag also co-immunoprecipitated with Apaf-1(3+4)-Myc as well as CED-4-Myc (Fig. 2*D*, *left* and *middle*). Previous studies had shown that epitope-tagging Bcl-X_L at the NH_2 terminus disrupts its ability to interact with CED-4 and this similarly inhibited binding to Apaf-1 (Ref. 9; Fig. 2*D*, *left*). A previously characterized dicodon mutant form of Bcl-X_L (mt7) that does not inhibit cell death did not bind Apaf-1, while an alternate dicodon mutant form Bcl-X_L (mt1), which blocks apoptosis but

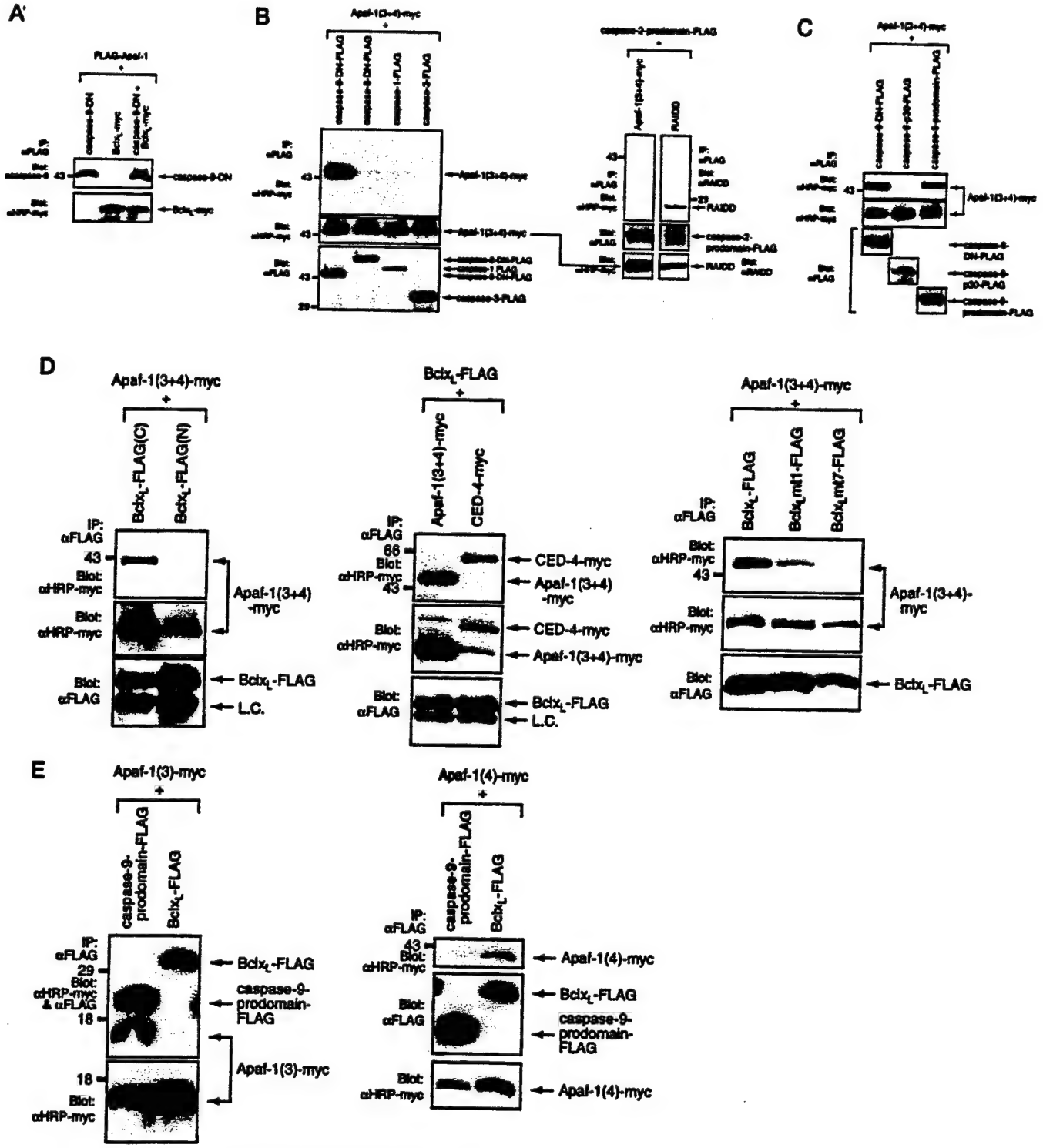

FIG. 2. **Both caspase-9 and Bcl-X_L co-immunoprecipitate with Apaf-1.** *A*, Apaf-1 binds caspase-9 and Bcl-X_L. 293 cells were transfected with indicated expression constructs for Flag-Apaf-1, caspase-9-DN, and Bcl-X_L-Myc (18, 19). After 36–40 h, extracts were prepared and immunoprecipitated (*IP*) with anti-Flag M_2 affinity gel (Kodak Scientific Imaging Systems). The presence of caspase-9, Bcl-X_L-Myc, and Flag-Apaf-1 was detected by immunoblotting with polyclonal antibody against caspase-9, horseradish peroxidase (*HRP*)-conjugated anti-Myc (*BMB*), and anti-Flag (Babco), respectively, as indicated. *B*, caspase-9, but not other caspases, interacts with Apaf-1. 293 cells were co-transfected with Apaf-1(3+4)-Myc and various caspase constructs as described under "Experimental Procedures." Cell lysates were co-immunoprecipitated with anti-Flag affinity gel, and immunoblotting was performed with various monoclonal antibodies (mAb) or polyclonal anti-RAIDD as indicated. The *middle* and *bottom panels* show the expression of the individual proteins. *C*, the prodomain of caspase-9 binds Apaf-1. Apaf-1(3+4)-Myc was co-expressed in 293 cells with various caspase-9 constructs as described under "Experimental Procedures." Cell lysates were co-immunoprecipitated with anti-Flag affinity gel, and immunoblotting was done with mAbs as indicated. *D*, Bcl-X_L binds Apaf-1. Apaf-1(3+4)-Myc was co-expressed with either COOH-terminal-tagged Bcl-X_L (*Bcl-X_L-FLAG(C)*) or NH_2-terminal tagged Bcl-X_L (*Bcl-X_L-FLAG(N)*). Co-immunoprecipitation was done with anti-Flag affinity gel, and immunoblotting was performed with various mAbs as shown. *L.C.*, light chain. *E*, caspase-9 and Bcl-X_L bind distinct regions in Apaf-1. Either caspase-9-prodomain-Flag or Bcl-X_L-Flag was co-transfected with Apaf-1(3)-Myc that contains only the CED-3-homologous region and Apaf-1(4)-Myc that contains only the CED-4-homologous region as described under "Experimental Procedures." Cell extracts were prepared and co-immunoprecipitated with anti-Flag affinity gel. The presence of each protein was detected by immunoblotting with various antibodies as indicated.

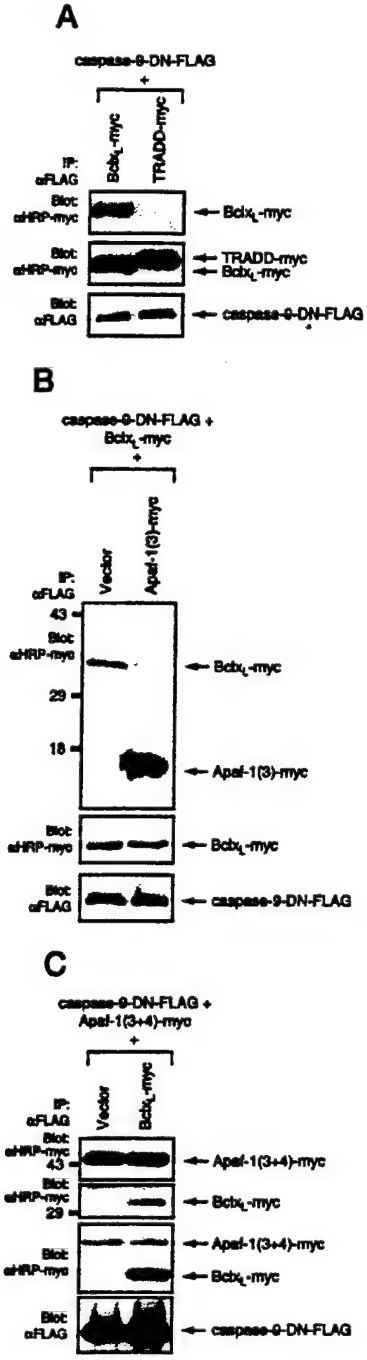

FIG. 3. **Caspase-9 and Bcl-X_L form a ternary complex with Apaf-1.** *A*, caspase-9 associates with Bcl-X_L through an endogenous Apaf-1-like activity. 293 cells were co-transfected with caspase-9-DN together with Bcl-X_L-Myc or a control molecule TRADD-Myc as indicated. Cell lysates were prepared and co-immunoprecipitated with anti-Flag affinity gel, and individual protein was detected by immunoblotting with the indicated mAbs. *B*, co-expression of the CED-3-homologous region of Apaf-1 disrupts the association of caspase-9 with Bcl-X_L. 293 cells were co-transfected with caspase-9-DN-Flag and Bcl-X_L-Myc in the presence of a vector or a construct expressing Apaf-1(3)-Myc. Co-immunoprecipitation was performed with anti-Flag affinity gel, and immunoblotting was done as indicated. *C*, Bcl-X_L does not affect the interaction between caspase-9 and Apaf-1. Caspase-9-DN-Flag was co-expressed with Apaf-1(3+4)-Myc in the presence of either a vector or a Bcl-X_L-Myc construct. Co-immunoprecipitation was done with anti-Flag affinity gel and proteins were detected by immunoblotting as indicated.

does not heterodimerize with other Bcl-2 family members (29, 30), retained binding to Apaf-1, albeit to a lesser extent (Fig. 2*D*, *right*). Regardless, the data are consistent with the notion that Bcl-X_L may function by interacting with Apaf-1.

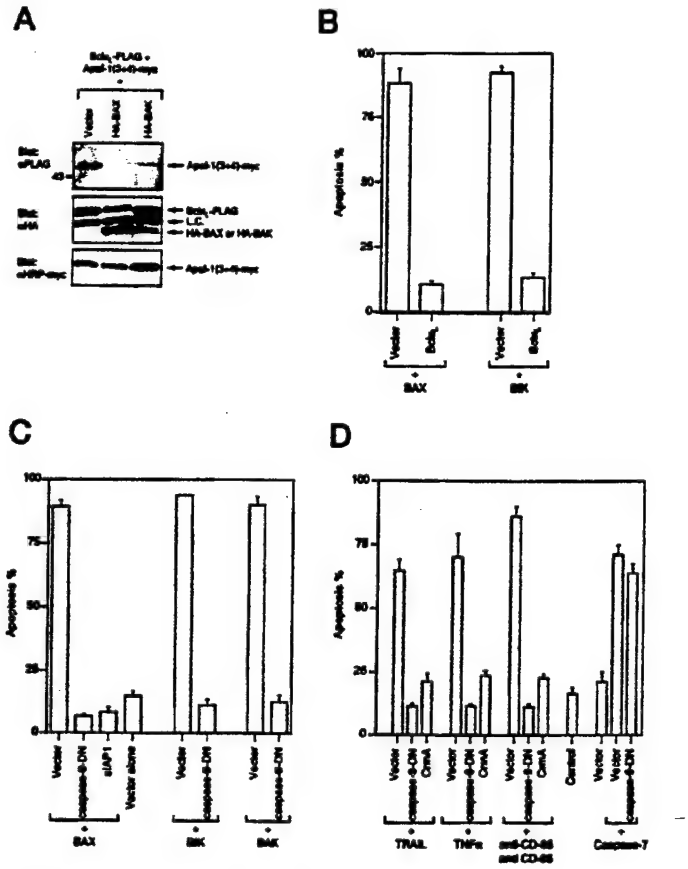

FIG. 4. **Caspase-9-DN blocks cell death induced by different death inducers.** *A*, BAX or BAK disrupts the interaction between Apaf-1 and Bcl-X_L. Bcl-X_L-Flag and Apaf-1(3+4)-Myc were co-expressed in the presence of either vector, HA-BAX or HA-BAK. Co-immunoprecipitation and immunoblotting were performed with antibodies as indicated. *B*, Bcl-X_L inhibits apoptosis induced by BAX or BAK in MCF7 cells. The apoptosis blocking assay was done and the data presented as in Fig. 1*B*. *C*, caspase-9-DN blocks cell death induced by BAX, BIK, or BAK. The cell death assay was done and data presented as in Fig. 1*B*. *D*, caspase-9-DN inhibits apoptosis initiated by TRAIL, TNFα, and agonist anti-CD-95. To determine the effect of caspase-9-DN on CD-95-induced apoptosis, a limited amount of CD-95 expression construct was co-transfected with either vector, caspase-9-DN, or CrmA. Twenty-four hours later, cells were treated with CD-95 antibody (1 mg/ml) to induce apoptosis. In other instances following transfection with the indicated plasmids, the cells were treated with either TRAIL or TNFα (40 ng/ml). Cells were stained and examined 4–6 h later and data presented as in Fig. 1*B*.

Caspase-9 and Bcl-X_L Bind to Distinct Regions in Apaf-1—To determine if caspase-9 and Bcl-X_L bind to distinct domains in Apaf-1 (3+4), the CED-3-homologous region alone (Apaf-1(3)) and a truncated form that contained only the CED-4-homologous region (Apaf-1(4)) were assessed separately for binding in a co-transfection assay. Caspase-9 bound the CED-3-homologous region (Apaf-1(3); Fig. 2*E*, *left*), while Bcl-X_L interacted with the CED-4-homologous region (Apaf-1(4); Fig. 2*E*, *right*). Therefore, caspase-9 and Bcl-X_L bind to distinct domains in Apaf-1, raising the possibility that they can form a ternary complex with Apaf-1.

Caspase-9 and Bcl-XL Form a Ternary Complex with Apaf-1—To assess this, we asked whether caspase-9 and Bcl-X_L might co-precipitate through an endogenous Apaf-1-like activity. 293 cells were co-transfected with caspase-9-Flag and Bcl-X_L-Myc. Caspase-9 co-precipitated with Bcl-X_L but not a control protein, TRADD-Myc (Fig. 3*A*). To confirm that the observed association was indeed mediated by an endogenous Apaf-1-like molecule, the CED-3-homologous domain of Apaf-1 (Apaf-1(3)-Myc) was co-expressed in the same cells. We reasoned that this domain, when present in excess, should com-

petitively inhibit the binding of caspase-9 to endogenous Apaf-1, thereby disrupting the association between caspase-9 and Bcl-X_L if the bridging molecule was indeed Apaf-1. As anticipated, Apaf-1(3)-Myc on co-expression attenuated the association of caspase-9-Flag and Bcl-X_L-Myc (Fig. 3*B*). Furthermore, Apaf-1(3)-Myc was observed in complex with caspase-9-Flag (Fig. 3*B*), confirming the competitive nature of the inhibition. Additional validation for ternary complex formation was provided by the observation that overexpressing Bcl-X_L-Myc in the same cells did not compete for the association of caspase-9 with Apaf-1(3+4)-Myc (Fig. 3*C*). This result is consistent with the existence of independent binding sites on Apaf-1 for caspase-9 and Bcl-X_L.

BAX and BAK Disrupt Interaction between Bcl-X_L and Apaf-1—The anti-apoptotic ability of Bcl-X_L is antagonized by pro-apoptotic members of the Bcl-2 family, including BAX, BAK, and BIK that are capable of forming heterodimers with Bcl-X_L (29, 30). Given this, we asked if the pro-apoptotic family members may function by interfering with the ability of Bcl-X_L to bind Apaf-1. In keeping with this hypothesis, co-expression of HA-BAX or HA-BAK attenuated the interaction between Bcl-X_L-Flag and Apaf-1(3+4)-Myc (Fig. 4*A*), with HA-BAX or HA-BAK being found in complex with Bcl-X_L-Flag (Fig. 4*A*). Consistent with the suggested mechanism, Bcl-X_L effectively inhibited BAX-, BIK-, or BAK-induced cell death (Fig. 4*B*). Since CED-9 functions upstream of CED-4 and CED-3, Bcl-2 family members likely also function upstream of Apaf-1 and caspase-9 (5). Supporting this viewpoint, we found that cell death induced by BAX, BIK, or BAK was effectively inhibited by dominant negative caspase-9 (Fig. 4*C*).

Dominant Negative Caspase-9 Inhibits Cell Death Initiated by Death Ligands and Agonist CD-95 Antibody—In agreement with the notion that activation of caspase-9 serves as a common conduit for the flow of death signals, dominant negative caspase-9 also blocked apoptosis induced by members of the TNF receptor family activated with either cognate ligand or agonist antibody (Fig. 4*D*).

In conclusion, we have shown that both caspase-9 and Bcl-X_L specifically and simultaneously interact with Apaf-1. Therefore, the formation of a complex involving caspase-9, Apaf-1, and Bcl-X_L may play a regulatory role in modulating the mammalian cell death machine.

Acknowledgments—We thank H. Duan for providing the caspase-9 p30-Flag construct; A. M. Chinnaiyan, E. Humke, J. McCarthy, and C. Vincenz for helpful discussion; I. Jones for preparing the figures; and B. Schumann for secretarial assistance.

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Chapter 4

MICE, a novel short prodomain caspase

Summary

Caspases are a family of cysteine proteases related to interleukin-1 converting enzyme (ICE) and represent the effector arm of the cell death pathway. The zymogen form of all caspases is composed of a prodomain plus large and small catalytic subunits. Herein we report the characterization of a novel caspase, MICE, (for mini-ICE) that possesses an unusually short prodomain and is highly expressed in development but is absent from all adult tissues examined. In contrast to the other short prodomain caspases (caspase-3, caspase-6, and caspase-7), MICE preferentially associates with large prodomain caspases, including caspase-1, caspase-2, caspase-4. Also unlike the other short prodomain caspases, MICE was not processed by multiple death stimuli including activation of members of tumor necrosis factor receptor family and expression of proapoptotic members of the bcl-2 family. Surprisingly, however, overexpression of MICE itself induced apoptosis in MCF7 human breast cancer cells that was attenuated by traditional caspase inhibitors.

Major advances have been made towards understanding the molecular mechanism of programmed cell death (1). Functioning as central components of the cell death signaling pathway are a rapidly growing family of cysteine proteases that cleave following aspartate residues (caspases) (2, 3). caspases are normally present as single polypeptide zymogens and contain an amino-terminal prodomain, and large (p20) and small (p10) catalytic subunits (4-6). The two chain active enzyme is obtained following proteolytic processing at internal Asp residues (4-6). As such, caspases are capable of activating each other in a manner analogous to the processing of zymogens observed in the coagulation cascade.

To date, twelve caspases have been identified and they can be classified into three subfamilies: caspase-1 (interleukin-1 converting enzyme), caspase-4 (ICErelII, TX, ICH2), caspase-5 (ICErelIII, TY), caspase-11 (Ich-3), and caspase-12 belong to the caspase-1 subfamily, caspase-2 (Ich-1) is the sole member of caspase-2 subfamily whereas caspase-8 (FLICE, MACH, Mch5), caspase-9 (ICE-LAP6, Mch6), caspase-10 (FLICE2, Mch4), caspase-3 (Yama, CPP32, apopain), caspase-7 (ICE-LAP3, Mch3, CMH-1) and caspase-6 (Mch2) belong to the caspase-3 subfamily (2, 3, 7). An alternate classification is based on the size of the prodomain as large prodomain caspases function as upstream signal transducers whereas short prodomain caspases function as downstream amplifiers that cleave death substrates (8). It is not entirely clear how large prodomain caspases are activated. However, recent studies suggest that their binding to receptor-associated adaptor molecules results in their approximation and activation by auto-processing (8-12).

Three short prodomain caspases exist in the caspase-3 subfamily , whereas none have been found in the other two subfamilies (2, 3). Here, we report a novel developmentally regulated short prodomain caspase that is a member of the caspase-1 subfamily and possesses unique biochemical properties.

Materials and Methods

Cell Lines and Expression Vectors - Human embryonic kidney 293 and 293 EBNA cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, nonessential amino acids, L-glutamine, and penicillin/streptomycin. Human breast carcinoma MCF7 cells were maintained in RPMI-1640 containing 10% heat-inactivated fetal bovine serum, nonessential amino acids, L-glutamine, and pennicillin/streptomycin. Expression constructs of tumor necrosis factor receptor family members were in pFLAG-CMV-1 (Invitrogen). Bax, Bak, Bik expression constructs were generously provided by G. Chinnadurai, IAP1 and IAP2 by D. V. Goeddel, and Hrk by G. Nunez. All other expression constructs were made in pcDNA3 (Invitrogen). Epitope tags were placed at the C-termini unless otherwise indicated.

Cloning of MICE - cDNA sequences corresponding to the partial open reading frame of MICE were identified as expressed-sequence tags (EST)(Genbank numbers AA103647 and AA167930) homologous to caspase family members. Both clones were sequenced using plasmid DNA template by the dideoxy chain termination method employing modified T7 DNA polymerase (Sequenase, U.S. Biochemical Corp.)

Northern blotting - Mouse adult multiple tissue and embryo tissue poly $(A)^+$ RNA blots were obtained from Clontech and processed according to the manufacturer's instructions. A ^{32}P-labeled cDNA corresponding to MICE amino acid residue 44-152 was used as probe.

Transfection, Coimmunoprecipitation and Western Analysis - Transient transfections of 293 cells were performed as described previously (13). Cells were harvested 20-30 hours following transfection, and either immunoprecipitated and immunoblotted or directly blotted with the indicated antibodies.

Cell Death Assay - 293 EBNA cells and MCF7 cells were transiently transfected with 0.1 and 0.25 μg of the reporter plasmid pCMV β-galactosidase respectively plus 0.5-1.0 μg of test plasmids in the presence or absence of 2.0 μg of inhibitory plasmids. 24-30 hours following transfection, cells were fixed with 0.5 % glutaraldehyde and stained with 5-bromo-4-chloro-3-indoyl β-D-galactoside. Percentage of apoptotic cells was determined by calculating the fraction of membrane blebbed blue cells as a function of total blue cells. All assays were evaluated in duplicate and the mean and standard deviation calculated.

Results and Discussion

MICE is a short prodomain caspase homologous to caspase-2

Analysis of the full length cDNA sequence of MICE revealed a 774-base pair open reading frame that encoded a novel protein of 257 amino acids with a predicted molecular mass of 29.5 kDa (Figure 1 A). Given the presence of an in-frame stop codon 12 base pairs upstream of the ininiator methionine (TGA AAG CCA GAC ATG GAG) and the fact that the putative initiator methionine was in agreement with the consensus Kozak's sequence for translation initiation, it was likely that the full length coding sequence was identified. Comparison of this protein with all known caspases revealed that it had a unusually short prodomain of only seven amino acids (Figure 1 &2). Given this, the molecule was termed MICE (for mini-ICE).

Phylogenetic analysis revealed MICE to be most related to caspase-1 subfamily members and it is therefore the first short prodomain caspase to be part of the caspase-1 subfamily (Figure 1 B). Overall, MICE displayed 21.4%, 19.5%, and 20.2% identity to the known short prodomain caspases 3, 6, and 7, respectively (Figure 2). The QACRG pentapeptide motif present in most caspases is also conserved in this novel caspase. In addition, based on the x-ray crystal structure of caspase-1 and caspase-3, amino acid residues involved in catalysis are conserved in MICE as are residues that form a binding pocket for the carboxylate side chain of the P1 aspartic acid (Figure 2)(4-6). This is in keeping with MICE being a functional caspase.

Tissue distribution of MICE- Mouse adult and embryonic tissue poly $(A)^+$ RNA blots were probed with a ^{32}P-labeled cDNA corresponding to the large catalytic subunit of MICE. A single transcript of 2.8 kilobases was observed (Figure 3). Unlike all known caspases that are expressed in both adult and embryonic tissues (7, 10, 11, 14-18), MICE was highly expressed in certain stages of embryonic development but was undetectable in all adult tissues examined, including heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis. Interestingly, the expression level of MICE appeared to increase during the later stages of development (the attenuated expression in day 11 may be due to lower loading of mRNA). The original EST clones were derived from murine embryo and adult skin tissue. Due to the unavailability of mouse skin and embryo tissue blots, we do not known if MICE is indeed expressed in skin and which embryo tissue(s).

MICE preferentially associates with certain large prodomain caspases- Since small prodomain caspases function downstream of large prodomain caspases, we asked if MICE being a short prodomain caspase bound any of the putative upstream large prodomain caspases. Surprisingly, MICE associated with most large prodomain caspases, including caspase-1, 2, 4, 8 and 10 (Figure 4). The other short prodomain caspases incuding caspase-3, caspase-6, and caspase-7 associated only with caspase-10 (data not shown). MICE did not bind the other short prodomain caspases and displayed only weak self-association in contrast to caspase-3 and other caspases which strongly self-associated (Figure 4 and unpublished data). The preferential dimerization with large prodomain caspases and weak self-association suggest that MICE may function through heterodimerization.

MICE is not processed in multiple death signaling pathways- Apoptosis can be efficiently induced by overexpressing death signaling receptors belonging to the tumor necrosis factor receptor (TNFR) family, including Fas/CD95, TNFR1, DR3, DR4, and DR5 (19-22). Overexpression of caspases, or pro-apoptotic members of bcl-2 family is an alternate means to induce rapid apoptosis (2, 3, 23).

Previous studies shown that caspase-3 and caspase-7 are processed following activation of TNFR1 and CD95 (14, 24). To further characterize MICE, we asked if it was processed on activation of these receptors. 293 cells were transiently transfected with expression constructs encoding death signaling receptors and MICE, or the three known short prodomain caspases: caspase-3, 7, and 6. Interestingly, all three known short prodomain caspases were processed on co-expressing the death signaling receptors (Figure 5). MICE, however, was not processed, suggesting that it is not involved in the death pathway engaged by these proapoptotic receptors (Figure 5).

Bax, Bak, Bik, Bad, Bid, and Hrk are proapoptotic members of bcl-2 family (23, 25). 293 cells were transiently transfected with expression contructs encoding short prodomain caspases and pro-apoptotic bcl-2 family members. In keeping with the prior results, all three known short prodomain caspases were processed on co-expression, but MICE was not processed (Figure 6).

Since one of the ESTs was derived from an adult murine skin library, we tested if MICE was processed in ultraviolet (UV) irradiation-induced cell death. 293 cells transfected with MICE were subjected to different doses of UV and the cleavage of MICE was assayed. 293 cells underwent extensive death under the irradiation of 600 Joues per m^2, however, MICE

was not found to be processed under any of these doses (figure 7). Three other caspases tested, including caspase-3, 7, and 1, were not processed either (Figure 7). It is believed that UV-induced apoptosis is Fas-dependent and caspase-3 and 7 were involved in Fas killing (24, 26). These were contradict with our findings. Consistent with our findings, the UV-induced apoptosis in thymocytes from DN-FADD transgenic mice was not abrogated, suggesting the existence of non-Fas pathway in UV-induced death signaling (27).

Since MICE was not processed on activation of a number of distinct physiologically relevant death pathway, we asked if it could serve as a substrate for known caspases. Expression constructs encoding MICE and known caspases were coexpressed in the presence or absence of the death signaling receptor, TNFR1. Consistent with previous results, no processing of MICE was observed despite the additional death signal from TNFR1 (Figure 8). MICE was also not processed by caspase-1 or 4, both members of the caspase-1 subfamily (data not shown). The failure of processing of MICE suggests that it likely functions in a very specific pathway that remains to be defined.

MICE induced-apoptosis is attenuated by inhibitors of apoptosis- To determine if MICE plays a role in cell death, 293 EBNA and MCF7 cells were transfected with expression plasmids encoding wild type MICE, a mutant version of MICE missing the p10 subunit, and caspase-8 as a positive control that has previously been shown to potently induce apoptosis in both cell lines (9, 10). Like the three other known short prodomain caspases, MICE had little effect on 293 EBNA cells (Figure 9 and unpublished data). However, it induced apoptosis in MCF7 cells (Figure 9). As expected, catalytically inactive MICE displayed substantially less death inducing activity. More importanly, MICE induced-apoptosis in MCF7 was inhibited by the baculoviral encoded inhibitors of apoptosis 1 and 2 (IAP1 and IAP2), and the broad spectrum baculoviral caspase inhibitor p35, but not by CrmA, MC159, or I-FLICE (Figure 9). CrmA is a cowpox serpin that inhibits caspase-1 and 8 activity while MC159 is a death effector domain containing decoy molecule encoded by molluscum contagiosum virus (28-29). I-FLICE is a naturally occurring catalytically inert dominant negative caspase (30). These inhibitors function at the apex of the apoptotic cascade by disrupting assembly of receptor signalling complexes and /or inhibiting the initiating caspase (29-31). Since they had no effect on cell death induced by MICE, it is likely that MICE function as a downstream signal transducer of cell death.

In summary, the failure of MICE to undergo processing in multiple known death pathways and its ability to physically interact with large prodomain caspases and induce cell death

suggests that MICE likely functions as a downstream active caspase in an unidentified signaling pathway.

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A

MESEMSDPQPLQEERYDMSGARLALTLCVTKAREGSEVDMEALERMFRYLKFESTMKRDPTAQQFLEELD
EFQQTIDNWEEPVSCAFVVLMAHGEEGLLKGEDEKMVRLEDLFEVLNNKNCKALRGKPKVYIIQACRGEH
RDPGEELRGNEELGGDEELGGDEVAVLKNNPQSIPTYTDTLHIYSTVEGYLSYRHDEKGSGFIQTLTDVF
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B

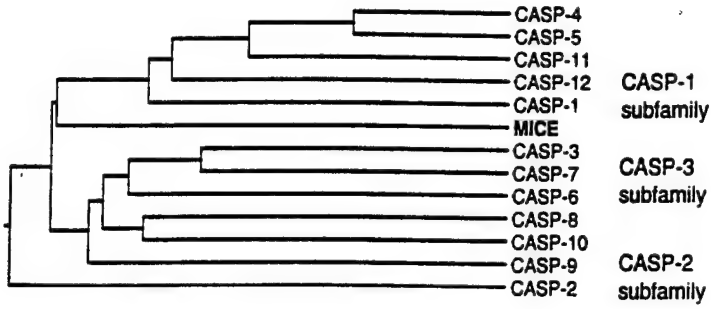

Figure 4. 1 Sequence of MICE. Deduced amino acid sequence of MICE. The conserved pentapeptide QACRG is boxed and the putative cleavage site between prodomain and p20 is underlined.

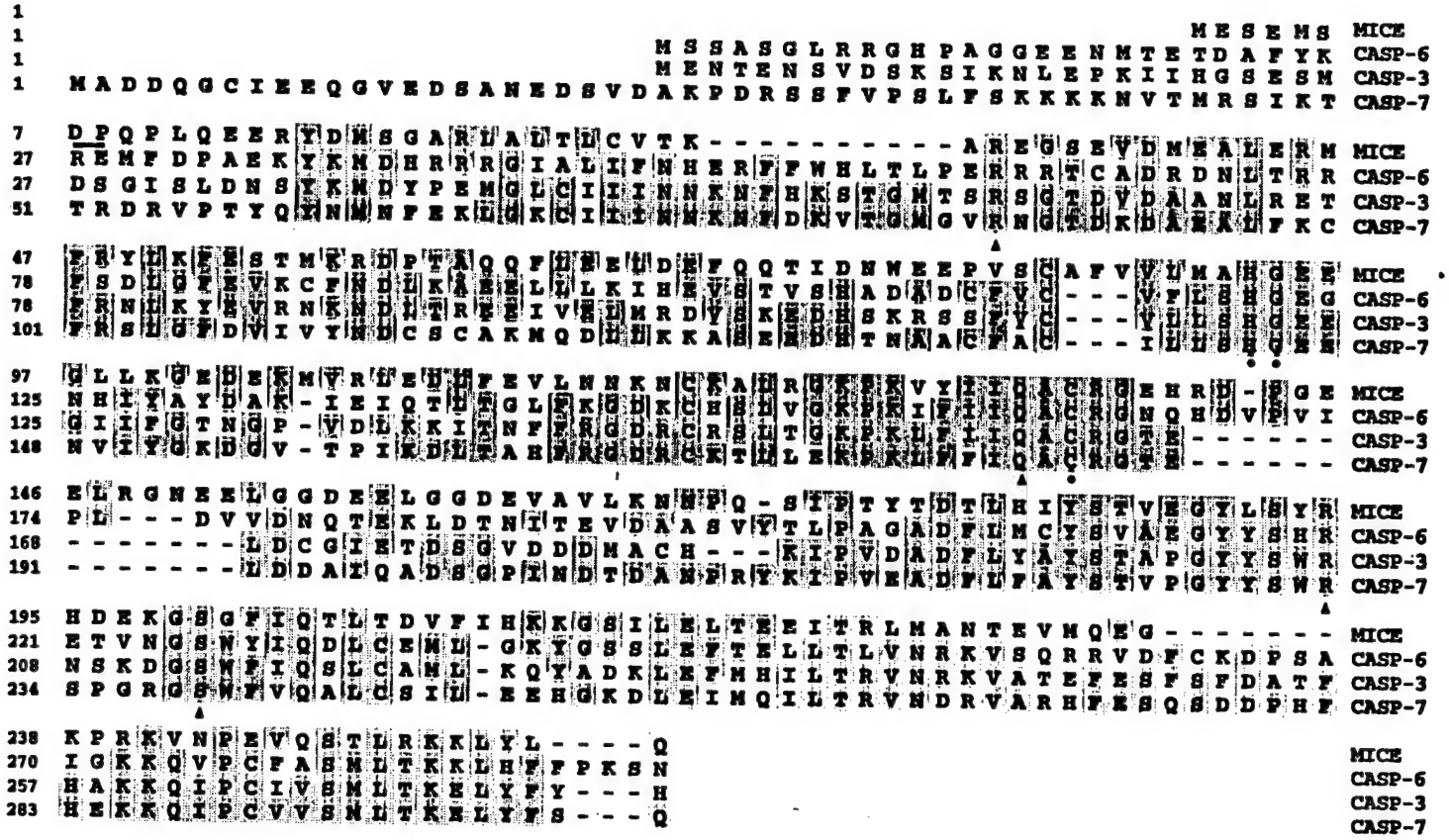

Figure 4. 2 Sequence alignment of MICE with known short prodomain caspases. Sequence alignment of MICE and three known short prodomain caspases. • indicate residues involved in catalysis and ▲ identifies residues that form the binding pocket for the carboxylate side chain of P1 Asp. The putative cleavage sites between prodomain and p20 are underlined.

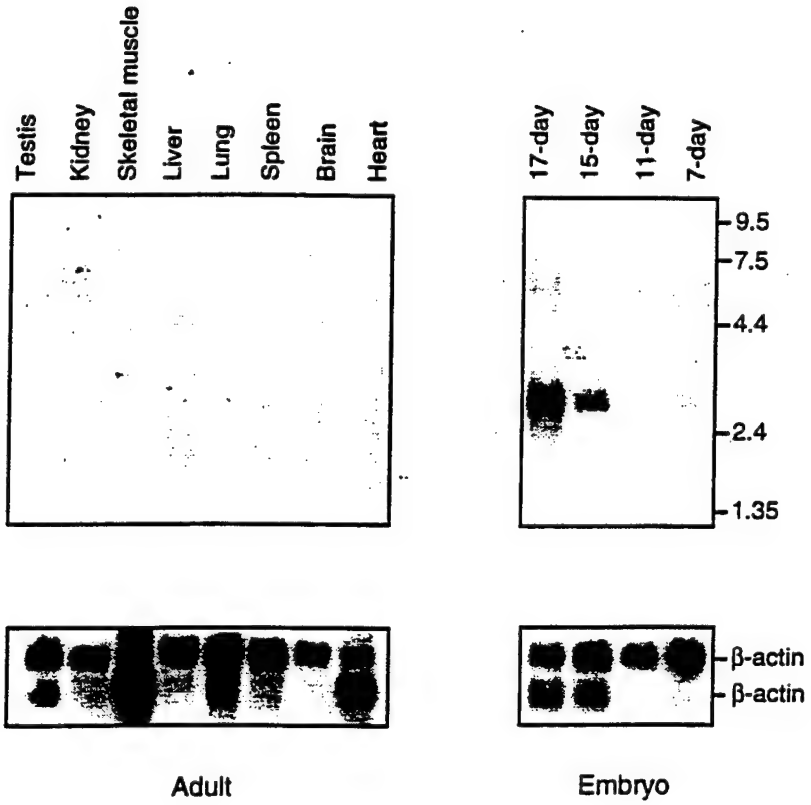

Figure 4. 3 Tissue distribution of MICE. Murine adult multiple and embryo tissue poly (A)+ Northern blots were probed with 32P-labeled MICE cDNA.

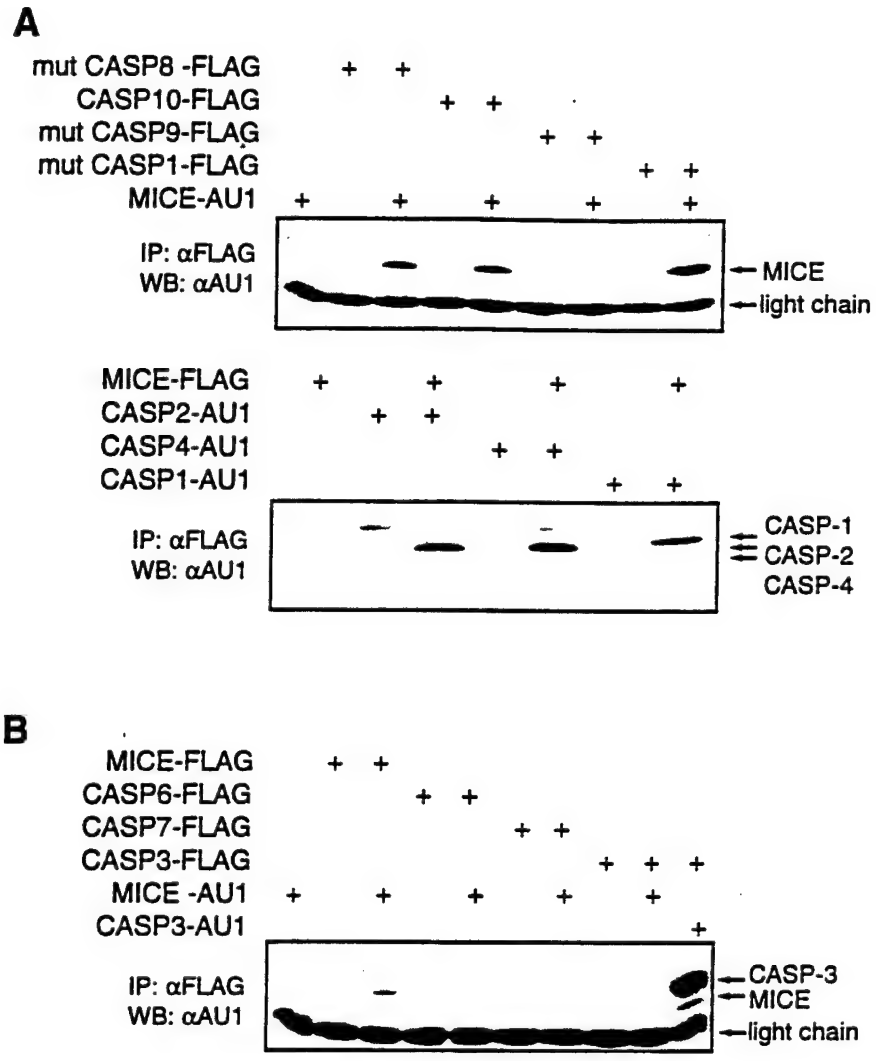

Figure 4. 4 Association of MICE with large prodomain caspases.293 cells were co-transfected with the expression constructs encoding MICE and other caspases. 30 hours following transfection, the cells were harvested, lysed, and analyzed with the indicated antibodies.

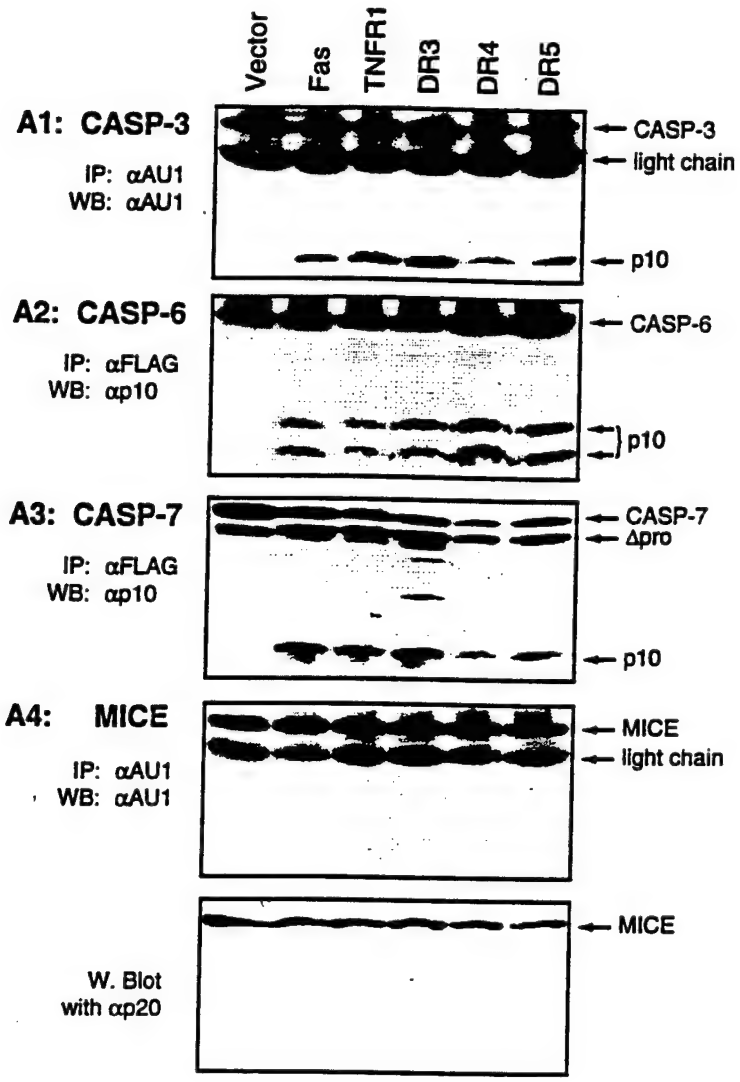

Figure 4. 5 MICE is not processed upon activation of death signaling members of TNFR family. 293 cells were co-transfected with expression constructs encoding TNFR family members and either caspase-3, caspase-6, caspase-7, or MICE. 20-24 hours following transfection, cells were harvested, and either immunoprecipitated and immunoblotted or directly immunoblotted with the indicated antibodies.

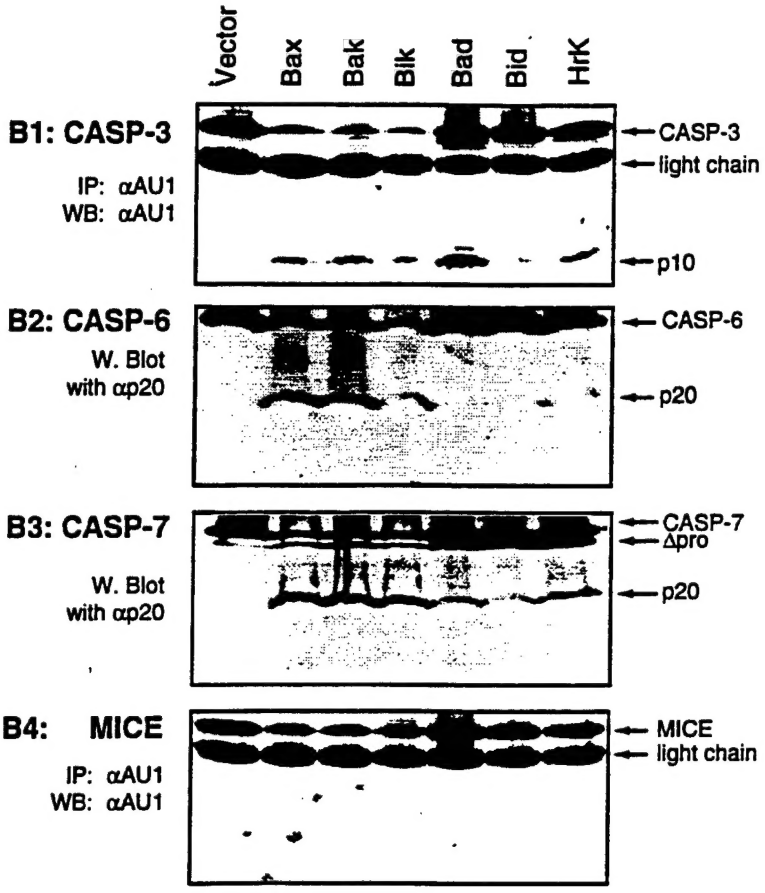

Figure 4. 6 MICE is not processed in death signaling pathways mediated by pro-apoptotic members of bcl-2 family. 293 cells were co-transfected with expression constructs encoding the pro-apoptotic members of bcl-2 family and either caspase-3, caspase-6, caspase-7, or MICE. 20-24 hours following transfection, cells were harvested, and either immunoprecipitated and immunoblotted or directly immunoblotted with the indicated antibodies.

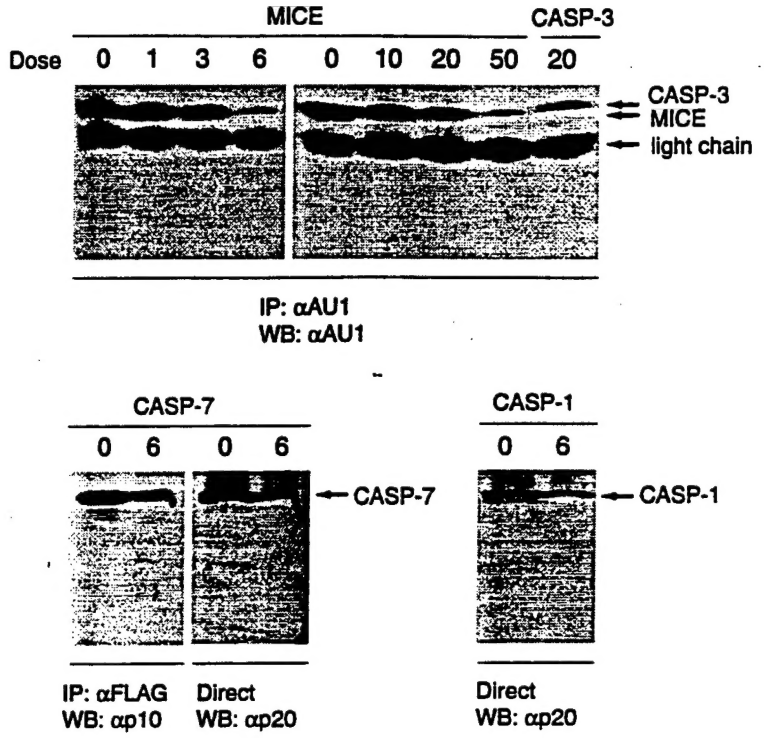

Figure 4. 7 MICE is not processed in ultraviolet irradiation-induced cell death pathway. 293 cells were co-transfected with expression constructs encoding MICE, caspase-3, or 7. 20 hours following transfection, cells subjected to different doses of UV irradiation. 18 hous after the treatment, cells were harvested, and either immunoprecipitated and immunoblotted or directly immunoblotted with the indicated antibodies.

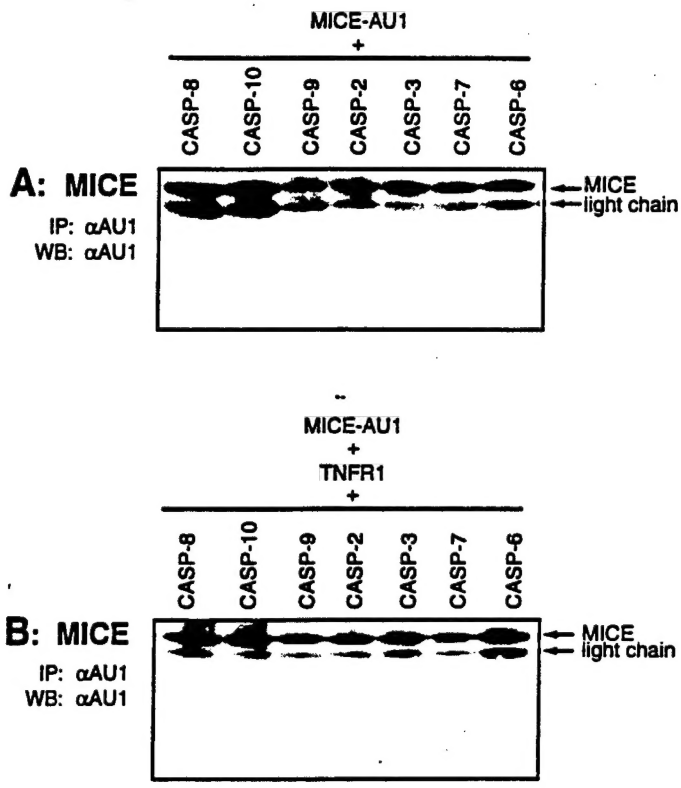

Figure 4. 7 MICE is not a substrate for known caspases.293 cells were co-transfected with expression constructs encoding MICE and caspases either in the absence or presence of TNFR1. 20-24 hours following transfection, cells were harvested, and either immunoprecipitated and immunoblotted or directly immunoblotted with the indicated antibodies.

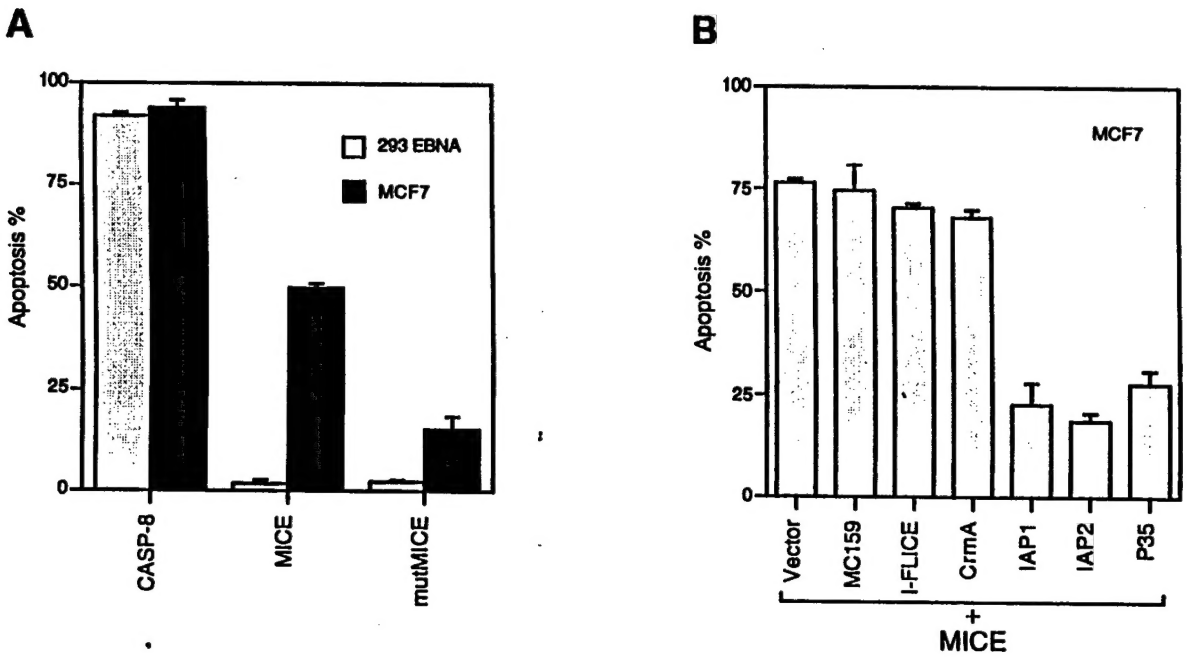

Figure 4. 9 MICE-induced apoptosis is attenuated by IAPs and p35. (A) 293 and MCF7 cells were co-transfected a reporter gene (b-galactosidase) and expression constructs encoding caspase-8, MICE, and a mutant version of MICE. (B) MCF7 cells were co-transfected the reporter gene and MICE expression construct and in the absence or presence of the inhibitor plasmids. The cells were fixed and stained as described in Materials and Methods.